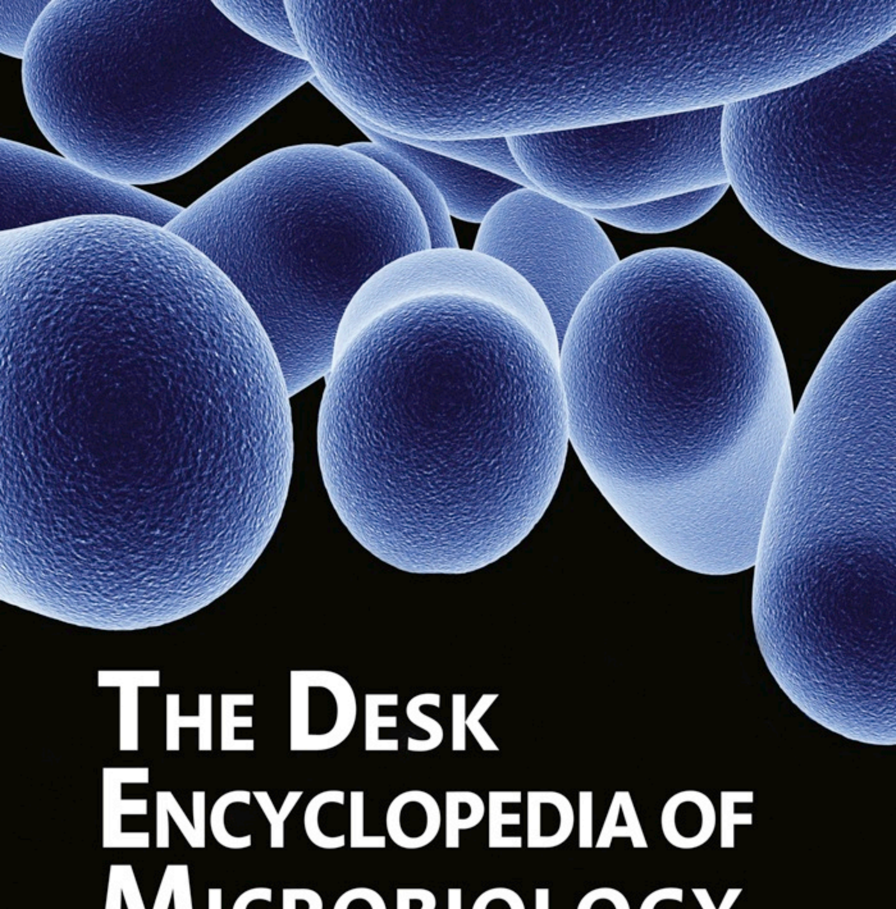

THE DESK ENCYCLOPEDIA OF MICROBIOLOGY

Second Edition

Edited by Moselio Schaechter

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DESK ENCYCLOPEDIA OF
MICROBIOLOGY

SECOND EDITION

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DESK ENCYCLOPEDIA OF MICROBIOLOGY

SECOND EDITION

EDITOR-IN-CHIEF

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PREFACE

The field of Microbiology encompasses highly diverse life forms – bacteria, archaea, fungi, protists, and viruses. Their influence on this planet is profound: they play an essential role in the cycles of matter in nature, affect all biological environments, interact in countless ways with other living beings, and play a crucial role in agriculture and industry. The field of Microbiology is so broadly encompassing that, of necessity, literature associated with it tends to be specialized and focused. For that reason, it is difficult to find sources that provide a broad perspective on a wide range of microbiological topics. This is the aim of *The Desk Encyclopedia of Microbiology*.

The purpose of this venture is to provide a single reference volume with appeal to microbiologists on all levels and fields, including those working in industry, health-related institutions, academia, and government. We believe that this book will be especially helpful for accessing material in areas in which the reader is not a specialist. It is intended to facilitate preparing lectures, grant applications and reports, and to satisfy curiosity regarding microbiological topics.

The Desk Encyclopedia of Microbiology is principally a synthesis from the comprehensive multi-volume *Encyclopedia of Microbiology*. Our intention is to provide affordable and ready access to a large variety of topics within one set of covers. To this end, we have chosen subjects that, in our opinion, will be of greatest interest to the largest number of readers. Included are the most general chapters from the *Encyclopedia of Microbiology*.

The result is a volume where coverage is extensive but not overly long in specific details. We believe this will be a most appropriate reference for anyone with an interest in the intriguing field of Microbiology.

Moselio Schaechter, 2009

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EDITOR-IN-CHIEF

Professor Schaechter did his graduate work at the University of Kansas and the University of Pennsylvania. He worked on the biology of rickettsiae at Walter Reed Army Institute of Research, and was a postdoctoral fellow for two years in Copenhagen, in the laboratory of Ole Maaløe. Professor Schaechter's research interest concerned various aspects of the regulation of bacterial growth. He discovered the existence of polyribosomes in bacteria and was among the first to elucidate aspects of polyribosome metabolism and the role of the cell membrane in DNA synthesis and chromosome segregation.

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Actinobacteria

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Defining Statement
Introduction
Systematics of *Actinobacteria*
Phylogeny of *Actinobacteria*
Genome Structure and Evolution
Industrially Important Phenotypes of *Actinobacteria*
Concluding Remarks
Further Reading

Glossary

co-metabolism The metabolic transformation of a substance by one organism to a second substance, which serves as a primary source of carbon for another organism.

mycelium The mass of hyphae that forms the vegetative and aerial parts of the streptomycete colony before sporulation.

phylogeny Evolutionary history of a group of organisms.

pseudogene A gene that has lost its protein-coding ability.

taxonomy Science of classification.

Abbreviations

ARDRA	Amplified ribosomal DNA Restriction analysis
CGH	comparative genomic hybridization
DF	dibenzofuran
DR	direct repeat
FAME	fatty acid methyl ester analysis
GITs	gastrointestinal tracts
HGT	horizontal gene transfer
hsp	heat shock protein
IS	insertion sequence
ISP	International *Streptomyces* Project
LFRFA	Low-frequency restriction fragment analysis
MLST	multilocus sequence typing
PAHs	polycyclic aromatic hydrocarbons
PCDOs	Polychlorinated dibenzo-*p*-dioxins
PCR-RAPD	PCR-randomly Amplified Polymorphic DNA
PFGE	pulse-field gel electrophoresis
PyMS	pyrolysis mass spectrometry
RFLP	restriction fragment length polymorphism
rRNA	ribosomal RNA
TEs	transposable elements
VNTRs	variable number tandem repeats

Defining Statement

The *Actinobacteria* form a distinct clade of Gram-positive bacteria which contains a large number of genera. This diverse and important group encompasses key antibiotic-producers, many soil bacteria critical for decomposition and resilient species capable of growing in hostile, polluted environments. A few are medically important pathogens including the causal agent of TB.

Introduction

Some of the earliest descriptions of *Actinobacteria* were those of *Streptothrix foersteri* in 1875 by Ferdinand Cohn and *Actinomyces bovis* in 1877 by Carl Otto Harz. Harz described *A. bovis*, causing a disease of cattle called 'lumpy jaw', as having thin filaments that ended in club-shaped bodies that he considered to be 'gonidia' resembling those of fungi, hence the name *Actinomyces* (Latin for ray fungus). In hindsight, though, the 'gonidia' were almost certainly host cells and so the resemblance to typical fungi was false. A number of other microorganisms were isolated, which had some of the same properties and were thought to belong to the same group as those mentioned above, including the causative agents of leprosy and tuberculosis. This group of organisms was officially recognized as *Actinomycetales* in 1916 and it became apparent that they comprised a large heterogeneous group, which vary greatly in their physiological and biochemical properties, though their phylogenetic position as true bacteria was not established until the 1960s. The *Actinobacteria* are now considered to be one of the largest phyla of the bacterial kingdom. The GC content of these organisms ranges from 54% in some corynebacteria to more than 70% in streptomycetes. *Tropheryma whipplei*, the causative agent of Whipple's disease, has a GC content of 46.3%, which is the lowest so far reported for *Actinobacteria*.

The *Actinobacteria* are morphologically diverse and can range from coccoids (*Micrococcus*) and rods (*Mycobacterium*) to branching mycelium (*Streptomyces*), many of which can also form spores. Actinobacterial species are ubiquitous in the environment and can be isolated from both aquatic and terrestrial habitats. New species of *Actinobacteria* have been recovered from a diverse range of environments, including medieval wall paintings, desert soil, butter, marine sponges, and radon-containing hot springs. The ability of *Actinobacteria* to inhabit varied environments is due to their ability to produce a variety of extracellular hydrolytic enzymes, particularly in the soil, where they are responsible for the breakdown of dead plant, animal and fungal material, thus making them central organisms in carbon recycling. Some species can break down more complex, recalcitrant compounds, of which *Rhodococcus* species are a good example; they can degrade nitro-, di-nitrophenol, pyridine, and nitroaromatic compounds.

Actinobacteria are well-known for their ability to produce secondary metabolites, many of which have antibacterial and antifungal properties. Of all the antibiotics produced by *Actinobacteria, Streptomyces* species are responsible for ~80%, with smaller contributions by *Micromonospora, Saccharopolyspora, Amycolatopsis, Actinoplanes*, and *Nocardia.* A number of species have developed complex symbiotic relationships with plants and insects. A unique relationship has been reported between the European bee-wolf wasp, in which the female wasps carry *Streptomyces* species in their antennae and apply them to the brood cells. The bacteria are taken up by the larva and colonize the walls of the cocoon, where they protect it from fungal infestation. *Streptomyces* species also share beneficial relationships with plants, with *S. lydicus* being found to enhance pea root nodulation by *Rhizobium* species. The best-studied example of a *Streptomyces*-eukaryotic relationship is between pathogenic strains such as *Streptomyces scabies* that cause scab in potatoes, carrots, beets, and other plants. Strains of *Frankia* can fix nitrogen and are responsible for the nodulation of many dicotyledonous plants. Some members of the *Actinobacteria* are important human and animal plant pathogens. These include *Mycobacterium leprae* (leprosy), *Mycobacterium tuberculosis* (tuberculosis in humans), *Mycobacterium bovis* (tuberculosis in cattle), *Corynebacterium diphtheriae* (diphtheria), *Propionibacterium acnes* (acne), and *Streptomyces somaliensis*, and *Actinomadura* and *Nocardia* species (actinomycetomas).

Systematics of *Actinobacteria*

Traditional Phenotypic Analysis

The best-studied members of the *Actinobacteria* class belong to the genus *Streptomyces*, which was proposed by Waksman and Henrici in 1943. Members of this genus have high GC content DNA, being highly oxidative and forming extensive branching substrate and aerial hyphae. They also produce a variety of pigments responsible for the color of the substrate and aerial hyphae (*Bergey's Manual of Systematic Bacteriology*). *Streptomyces* species are prolific producers of antibiotics, and since the discovery of actinomycin and streptomycin produced by *S. antibioticus* and *S. griseus* respectively during the early 1940s, the interest in streptomycetes grew very rapidly. The discovery of *Streptomyces* species as rich sources of commercially important antibiotics led to new techniques for the cultivation of these organisms. However, due to the lack of standards for the classification and identification of new species, the new strains were described based on only small differences in morphological and pigmentation properties. This, along with the belief that one strain produced only one antibiotic, led to overspeciation of the genus, resulting in over 3000 species being described by the late 1970s. A number of methods were developed to overcome this problem, with the earliest being based on only a few subjectively chosen characters focusing largely on morphological and pigmentation properties that were rarely tested under standardized conditions. Subsequently, biochemical, nutritional, and physiological characters were included, but as these were only applied to selected species they did not necessarily reflect the phylogeny of streptomycetes. The International *Streptomyces* Project (ISP) was established in 1964 with an aim to describe and classify extant type strains of *Streptomyces* using traditional tests under standardized conditions. This study resulted in more than 450 species being redescribed, but an attempt to delineate the genera was futile.

The data collected by the ISP project were used by several researchers to develop computer-assisted identification systems. In 1962, Silvestri was the first to apply numerical taxonomy to the genus *Streptomyces*, where nearly 200 strains were tested for 100 unit characters. This study highlighted the fact that many of the characters used to describe *Streptomyces* species are highly variable and can be erroneously interpreted. Williams and colleagues carried out a more comprehensive study of the streptomycetes. The majority of the strains studied were from the ISP project, along with soil isolates and representative strains from 14 other Actinobacterial genera. Each strain was tested for 139 unit characters, including spore chain morphology, spore chain ornamentation, color of aerial mycelium, color of substrate and extracellular pigments, production of extracellular enzymes, carbon and nitrogen source utilization, and resistance and sensitivity to certain antibiotics. Strains were clustered according to the observed similarities and this resulted in 19 major, 40 minor, and 18 single-strain clusters being identified, where the single-strain clusters were considered as species and the major clusters were referred to as species groups. The largest species group is cluster 1 *S. albidoflavus*, containing ~70 strains. This cluster is divided into three subclusters. Subcluster 1A contains strains like *S. albidoflavus, S. limosus*, and *S. fellus*, which form hooked or straight chains of smooth, yellow, or white spores and are melanin-negative. Subcluster 1B strains are similar in morphology and pigmentation to those in subcluster 1A and comprise strains such as *S. griseus, S. anulatus,* and *S. ornatus*. Strains in subcluster 1C produce gray, smooth spores and are melanin-negative; *S. olivaceus, S. griseolus*, and *S. halstedii* represent this group.

As in streptomycete taxonomy, early attempts to differentiate mycobacterial species were based on phenotypic properties such as growth rate and pigmentation. With the discovery of new species and the fact that pigment production can be temperature-dependent and that not all strains of a species share pigment-producing abilities, classification of mycobacterial species became less reliable. An alternative scheme was based on the pathogenic potential of a species, although this too was constantly changing as pathogenicity was being discovered in more species of *Mycobacterium.* The International Working Group on Mycobacterial taxonomy was set up in 1967 with an aim to standardize techniques used for the classification of these strains. This led to a numerical taxonomic approach to study mycobacterial strains. Closely related strains of *Corynebacterium, Rhodococcus*, and *Nocardia* were also included in these studies, which revealed that strains belonging to these four genera form distinct clades significantly separate from each other, with many strains being reclassified. Strains identified as *Corynebacterium equi* and *C. hoagii* were found to belong to *Rhodococcus*, and further chemical and genetic analysis confirmed the reclassification as they conformed to the original description of the genus. *Bacterionema matruchotti* was included in this study as its generic position was unresolved, being originally classified as a member of the Actinomycetaceae family. This strain was found to have all the characteristics of true corynebacteria and was renamed *Corynebacterium matruchotti.*

Attempts were made to resolve the confusion surrounding the classification of *Nocardia asteroides* and its relationship to *N. farcinica.* One hundred and forty-nine randomly selected *Nocardia* strains from various sources were analyzed. This study recovered seven major, nine minor, and twelve single-strain clusters. Two apparently identical strains of *N. farcinica* (NTCC 4524 and ATCC 3318) were found to group in two separate clusters. Strain NTCC 4524 clustered with *Mycobacterium* species, whereas 3318 grouped with *N. asteroides*. Numerical taxonomic studies have enabled the description of each species to be made and facilitated the development of new methods that would allow closely related species to be distinguished.

Characterization Based on Chemical Constituents

One of the drawbacks of numerical taxonomy is that it measures phenotypic similarities and differences and these do not always correlate with the genotype and thus only provide an estimate of relatedness between strains. Numerical taxonomy has largely been superseded by chemo- and molecular taxonomy. Chemotaxonomic methods have long been used to determine the relatedness between organisms. Goodfellow and colleagues used comparisons of fatty acid methyl ester analysis (FAME) between bacterial genera. Members of the Streptomycetaceae family have been described as having major amounts of either LL-diaminopimelic acid (LL-A_2pm) (*Streptacidiphilus* and *Streptomyces*) or *meso*-diaminopimelic acid (*meso*-A_2pm) (*Kitasatospora*) in their substrate mycelium and LL-A_2pm as the major diamino acid in aerial or submerged spores. Analysis of whole-cell sugar patterns revealed galactose or galactose and rhamnose (*Kitasatospora* and *Streptacidiphilus*). The presence of LL-A_2pm and glycine, with the absence of characteristic sugars, is typical of the *Streptomyces* cell wall type, which is characterized as Type I. These members also contain saturated iso- and anteiso- fatty acids with either seven or eight hydrogenated menaquinones with nine isoprene units as the predominate isoprenologues. They lack mycolic acids but contain the lipids phosphatidylglycerol, phosphatidylethanolamine, phophatidylinositol, and phosphatidylinositol mannosides. Chemotaxonomic characteristics of other families of the *Actinobacteria* class are summarized in **Table 1**.

Curie-point pyrolysis mass spectrometry (PyMS) has been applied for typing *Actinobacteria.* This method provides a fingerprint of the organisms, which can be used quantitatively to analyze differences between strains. Both FAME and PyMS require stringent standardization as changes in culture media and incubation can affect the results. Many examples exist in the literature where chemotaxonomy has been used successfully for the rapid characterization of new species as well as confirming the integrity of existing taxonomic clusters.

Genotypic Approaches to Determining Relatedness

DNA–DNA hybridization

Streptomyces coelicolor A3(2) and *S. lividans* 66 are members of the cluster 21 *Streptomyces violaceoruber* species group as defined by Williams and colleagues, which represents one of the well-defined species groups of the genus. Cluster 21

Table 1 Chemotaxonomic characteristics of selected families belonging to the class *Actinobacteria*

Family	*Phospholipid pattern*	*Fatty acids*	*Menaquinone*	*Diamino acid*	*Interpeptide bridge*	*Cell wall sugars*
Dermatophilaceae	PG, DPG, PI, PE, PC	$C_{16:0}$, $C_{15:0}$, $C_{14:0}$, $C_{17:0}C_{17:1}$ $C_{18:1}$	MK-8(H_4)	*meso*-A_2pm	None	
Dermacoccaceae	PG, DPG, PI, PE, PC	$C_{17:0}$, $C_{18:0}$, $C_{18:1}$, i-$C_{17:0}$i-$C_{17:1}$, ai-$C_{17:0}$	MK-8(H_4), MK-8(H_2), MK-8MK-9, MK-10	L-lysine	L-Ser$_{1\text{-}2}$-D-Glu/ L-Ser$_{1\text{-}2}$-L-Ala-D-Glu,D-Glu$_2$, L-Ser-D-Asp	
Cellulomonadaceae	PG, DPG, PI	ai-$C_{15:0}$, $C_{16:0}$	MK-9(H_4)	L-lysine/ornithine	L-Thr←D-Asp/L-Thr←D-Glu,D-Asp, D-Glu	Rhamnose
Micrococcaceae	CL, PG, DPG, PI, PL	i-$C_{15:0}$, ai-$C_{15:0}$,ai-$C_{17:0}$i-$C_{16:0}$	MK-7(H_2), MK-8(H_2), MK-9(H_2)MK-8, MK-9, MK-10	*meso*-A_2pm, Lysine LL-A_2pm, ornithine	L-lysine, L-alanine	Galactosamine, glucosamine
Corynebacteriaceae	DPG, PG, PI, PIM, PG	$C_{16:0}$, $C_{18:1}$, $C_{18:0}$	MK-8(H_2), MK-9(H_2)	*meso*-A_2pm		Arabinose/ galactose
Micromonosporaceae	PC, PE	i-$C_{15:0}$, i-$C_{15:1}$, $C_{17:1}$	MK-9(H_4), MK-10(H_4), MK-10(H_6) MK-12(H_4), MK-12(H_6), MK-12(H_8)	*meso*- or LL-A_2pm	L-glycine	Xylose/arabinose
Streptosporangiaceae	PG, PI, PE	i-$C_{16:0}$, $C_{17:0}$, i-$C_{18:0}$	MK9(H_4), MK-10(H_4)	*meso*-A_2pm		Madurose, glucose, ribose, mannose
Nocardioidaceae	PG, DPG, PE, PC PI, PIM	i-$C_{16:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ ai-$C_{15:0}$, i-$C_{14:0}$, $C_{18:1}$	MK-8(H_4), MK-9(H_4), MK-9(H_6) MK-9(H_8), MK-10(H_4)	LL-A_2pm	L-glycine	Glucose, ribose, mannose, galactose
Intersporangiaceae	PI, PIM, PG, DPG, PE	i-$C_{15:0}$, ai-$C_{15:0}$, i-$C_{14:0}$, i-$C_{16:0}$, $C_{17:0}$	MK-8, MK-8(H_4)	LL-A_2pm or *meso*-A_2pm	L-glycine	Glucose

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; PL, unknown phospholipids; MK, menaquinone; *meso*-A_2pm, *meso*-diaminopimelic acid; LL-A_2pm, LL-diaminopimelic acid. Taken from Dworkin *et al*., 2006.

strains produce smooth gray spores and diffusible pigments, which are blue or red depending on the pH of the medium. *S. coelicolor* A3(2) and *S. lividans* 66 are model representatives of this cluster as they have been genetically, biochemically, and physiologically characterized. However, both strains have had a long and confused taxonomic history.

In 1908, Muller isolated an actinomycete as a contaminant that produced a soluble blue pigment and named it *Streptothrix coelicolor*. In 1916, Waksman and Curtis independently isolated an actinomycete culture from the soil, which also produced a red and blue pigment and was named *Actinomyces violaceoruber*. For a long time the two strains were considered to be synonyms and when the *Streptomyces* genus was established both were named *S. coelicolor* (Muller) Waksman and Henrici in the fourth edition of *Bergey's Manual of Systematic Bacteriology*. Subsequently isolated strains that produced blue pigments were either considered to be *S. coelicolor* (Muller) Waksman and Henrici or as different species altogether. Kutzner and Waksman reexamined all the strains that produced a blue, red, and purple pigment and clarified that the strains isolated by Muller in 1906 (*S. coelicolor* (Muller)) and Waksman and Curtis in 1916 (*S. violaceoruber*) are distinctly different species. Analysis of the blue pigments produced by these strains showed that they are chemically very different. *S. coelicolor* (Muller) is a member of cluster 1 streptomycetes, showing similarity to *S. griseus* and is not a member of the *S. violaceoruber* clade. Monson and colleagues (1969) confirmed the results of Kutzner and Waksman by DNA–DNA hybridization between *S. violaceoruber* and *S. coelicolor* (Muller).

DNA–DNA hybridization experiments are an acknowledged approach in determining the integrity of taxonomic clusters defined by numerical taxonomy, and the study of Monson and colleagues (1969) was one of the first to use this technique. Research based on numerical phenetic and DNA–DNA hybridization data has revealed high levels of congruence as the same taxonomic groups are recovered. The cluster 18 *S. cyaneus* species group is highly heterogeneous, with 9 out of 18 type strains being assigned to two DNA relatedness groups defined at or above the 70% relatedness level. The use of DNA–DNA hybridization experiments also demonstrated that the cluster 32 *S. violaceusniger* species group encompasses several genomic species when type strains are examined. The DNA relatedness groups were defined at similarity levels >70%, seven of which consisted of single members. The multimembered clusters were equated with *S. hygroscopicus* and *S. violaceusniger*. The latter two species were redescribed and a number of strains carrying different specific names reduced to synonyms of the newly redescribed taxa. A high degree of heterogeneity in the *Streptomyces lavendulae* (cluster 61) cluster was reported by Labeda and Labeda and Lyons. A number of strains were related at the species level as they shared high DNA relatedness values (>80%). *S. colombiensis* was reduced to a synonym of *S. lavendulae* as it showed 83% DNA homology to *S. lavendulae* type strain. A number of strains showed <45% relatedness and were therefore considered to belong to a different species group. The DNA relatedness studies discussed above outline the importance of evaluating numerical taxonomic clusters using taxonomic criteria. This is particularly important in the case of the *Actinobacteria* that comprise many species. In situations such as this there is a risk of grouping unrelated or partially related strains together in clusters using insufficient properties.

Members of the *M. tuberculosis* complex include the strains *M. tuberculosis, M. bovis, M. africanum, M. canettii*, *M. microti*, *M. caprae*, and *M. pinnipedii*. DNA–DNA hybridization analysis reveals that this complex comprises a single species. Subsequent analysis of the genomes has revealed little sequence variation among the members. DNA–DNA hybridization has been used to study *Nocardia* species and this allowed the differentiation between species of *N. asteroides* and other members of the genus. Stackebrandt and Fiedler studied 16 strains of *Arthrobacter* and two strains of *Brevibacterium*. The DNA of these strains was hybridized to the *Arthrobacter* type strain *A. globiformis*. This analysis indicates that *Arthrobacter* species share little homology between themselves, with values ranging from 11 to 55%. However, *Brevibacterium sulfureum* and *Brevibacterium protophormiae* showed relatively high homology to the *Arthrobacter* strains. Based on these results it was recommended that the two *Brevibacterium* strains be reclassified as *Arthrobacter* strains. DNA–DNA hybridization is routinely used to aid the characterization of novel species of *Actinobacteria* with many examples in the literature.

Comparative genomic analysis

The availability of whole genome sequences has allowed the development of microarrays, which have revolutionized functional and genomic analysis of organisms. Microarrays have been extensively used to analyze gene expression and regulation and have application in a number of disciplines, including immunology, oncology, forensic science, pharmacogenomics, and drug discovery. More recently, microarrays have been used to investigate the genome-wide analysis between closely related strains, in particular those of pathogens, with an aim to identify pathogenicity determinants. This comparative genomic hybridization (CGH) microarray analysis has been used to investigate a number of bacterial pathogenic species in relation to pathogenesis and host specificity. To date, there are ~115 actinobacterial genomes that are either completed or at various stages of completion (www.genomesonline.org). This has led to the development of microarrays for some sequenced genomes, including those for *S. coelicolor*, and *Mycobacterium* and *Corynebacterium* species. To date, many of the microarray studies have focused on expression analysis of RNA, particularly with the pathogenic strains of *Mycobacterium* and *Corynebacterium*. Microarrays can also be used for DNA–DNA comparative genomic analysis between closely related strains, as was done for members of the cluster 21 *S. violaceoruber* strains. Using PCR-based microarrays Weaver and colleagues were able to identify 1-Mb amplification of the terminal regions of a number of laboratory strains of *S. coelicolor* A3(2) compared to the sequenced strain M145. The comparison of the cluster 21 streptomycetes also revealed 14 regions that were present in *S. coelicolor* M145 but absent in the other members. These regions encoded genes for biosynthesis of secondary metabolites and heavy metal resistance. All 14 regions were associated with transposon and insertion sequence (IS) elements and the fact they showed a much lower GC content than the rest of the *Streptomyces* chromosome strongly suggests these regions to have been acquired by horizontal gene transfer (HGT) by *S. coelicolor* M145. Ward and Goodfellow have reported a core set of genes from the comparative analysis of the Cluster 21 strains. These genes included those already identified as housekeeping genes and also those for some secondary metabolites; for example, genes for biosynthesis of actinorhodin were found to be conserved among the cluster 21 strains. Ul-Hassan (2006) used oligo-based microarrays for the comparative genomic analysis of soil strains identified as *S. violaceoruber*. The results revealed the strains to have undergone extensive deletions that could be correlated with their observed phenotypes. CGH microarray has been used to investigate the molecular taxonomy of a number of organisms, including *Saccharomyces* and *Yersinia* species and Clostridia. As more actinobacterial genomes are becoming available there is the potential to develop CGH microarray as a taxonomic tool to study environmentally and clinically important *Actinobacteria*.

Restriction digestion analysis of total chromosomal DNA

Restriction digestion analysis of total chromosomal DNA provides a fingerprint of the organisms being analyzed. Low-frequency restriction fragment analysis (LFRFA), restriction fragment length polymorphism (RFLP), and pulse-field gel electrophoresis (PFGE) have all been used to provide an indication of relatedness between strains. However, the results of these methods can be misleading if the strains have undergone large chromosomal deletions or amplifications. Amplified ribosomal DNA restriction analysis (ARDRA) requires the amplification of parts of the rRNA operon, including part of the 16S rRNA, the 16S–23S rRNA spacer region, and part of the 23S rDNA. The amplified PCR products are subjected to restriction enzyme digestion and electrophoresis, providing specific banding patterns for the strains being analyzed. ARDRA has been used to differentiate strains of *Arthrobacter* and *Microbacterium*. With the use of ARDRA in conjunction with PFGE, strain-specific restriction patterns for *Arthrobacter* and *Microbacterium* have been obtained. RFLP of the rRNA gene has been developed as a tool for identification of corynebacterial strains. The strains could be grouped based on the banding patterns and generally strains belonging to the same species clustered together. PCR-restriction enzyme pattern analysis (PRA) of Hsp65 (a heat shock protein) has been developed as a tool for differentiating between *Nocardia* species. However, when PRA analysis was applied to *Nocardia* species, the same banding patterns were recovered, demonstrating this technique not to be as useful for identification or differentiation of these species. Sequence analysis of the *Hsp65* gene displays sufficient polymorphic sites to allow identification. For methods which generate banding patterns that are subsequently used for identification, it is important to stress that a different banding pattern does not necessarily represent a new species. The differences may be attributed to genome rearrangements such as amplifications or deletions, which occur frequently.

PCR-randomly amplified polymorphic DNA (PCR-RAPD) involves PCR of the genome with an arbitrary set of primers to generate a characteristic fingerprint profile. The advantage of this method is that prior knowledge of the chromosomal sequence is not necessary, although stringent standardization is required. The drawback of this technique is that it is highly sensitive and variability in banding patterns may be observed depending on the type of reaction mixture, primers, and concentration of target DNA. This was used to analyze the relationship between *S. lavendulae* and *Streptomyces virginiae*, which were reported by Williams and colleagues to be synonyms. Although consistent results were obtained when comparing to DNA–DNA hybridization, LFRFA, and biochemical properties, the interspecific relationship of *S. lavendulae* and *S. virginiae* remained unresolved.

Analysis of the genomes of members of the *M. tuberculosis* complex demonstrates a number of repeat sequences, of which the best studied is the direct repeat (DR) region. This region consists of DR sequences (36 bp) interspersed with spacer sequences (34–41 bp), collectively termed direct variable repeat sequences. Spoligotyping was developed as a tool for analyzing the structure of the DR region. Spoligotyping patterns are produced by hybridization of sample DNA to oligonucleotides based on the specific sequences in the DR region. An international spoligotype database was set up and now consists of ~39 000 entries from research groups worldwide. The spoligotype patterns can be aligned, enabling researchers to group isolates based on these alignments into clades or strain families. Filliol and colleagues and Molhuizen and colleagues (1998) were able to identify distinct spoligotype patterns for nearly all of the defined *M. tuberculosis* complex strains. However it could only provide limited discrimination of *M. bovis* strains. Analysis of multiple genomic regions that contain variable number tandem repeats (VNTRs) of different families of genetic elements has been proposed as an alternative tool to examine *M. bovis* strains. The studies of Roring and colleagues and Allix and colleagues have shown VNTR to be more discriminatory than both spoligotyping and RFLP for *M. bovis*.

Phylogeny of *Actinobacteria*

Molecular Analysis of 16S rRNA Sequences

Molecular methods are now used together with numerical and chemotaxonomic techniques to improve the understanding of species relatedness. Woese and Fox used molecular systematic analysis of ribosomal RNA (rRNA) molecules to provide an evolutionary classification of organisms. All 16S rRNAs have conserved primary structures, which allowed Edwards and colleagues, to design universal primers to amplify the entire 16S rRNA gene. Analysis of the 16S rRNA gene revealed regions that are genus-specific and more variable regions that can be used to infer relationships at a lower taxonomic order. Stackebrandt and colleagues identified three regions within the 16S rRNA gene that show variation: α-region (nt 982–998) (*S. ambofaciens* nomenclature) and β-region (nt 1102–1122), which can be used to resolve species to the genus level. The most variable region is the γ-region (nt 150–200), which is species-specific. Inferred relationships based on gene sequences are subject to a number of assumptions with the major condition being that the gene analyzed must not be subject to gene transfer. It is well-known that bacteria contain multiple copies of the 16S rRNA gene but transfer of 16S rRNA has not been determined definitively. It has been suggested that high levels of similarity can facilitate recombination between closely related species, resulting in strains containing chimeric molecules of 16S rRNA. Phylogenetic analysis using 16S rRNA genes can pose problem due to intraspecific variation and intragenomic heterogeneity. Another problem with using 16S rRNA genes is that it is a slowly evolving gene, making it more difficult to resolve the relationships between strains to the species level. Katakoa and colleagues was able to examine the γ-region of a number of streptomycete strains and, although being too variable for determining generic relationships, the inter- and intraspecies relationships were resolved. When Hain and colleagues investigated the *S. albidoflavus* group it was apparent that use of the 16S rDNA sequences were useful for species delimitation but not strain differentiation. The 16S–23S intergenic region was better in determining intraspecific relationships. Based on the similarities of the 16S rRNA gene, Wellington and colleagues included *Kitosatospora* into the *Streptomyces* genus. This was contested by Zhang and colleagues, who demonstrated that strains of *Kitosatospora* always form a stable monophyletic clade away from the streptomycetes when the entire 16S rDNA sequences were used. Streptomycete-specific primers have also been designed for the 23S and 5S rRNA gene. Sequence analysis of the 5S rDNA gene sequences were used to confirm that *Chainia, Elytrosporangium, Kitasatoa, Microellobosporia*, and *Streptoverticillium* as members of the *Streptomyces* genus. Stackebrandt and colleagues proposed a new hierarchic system of classification for *Actinobacteria,* which was based exclusively on 16S rRNA-rDNA sequences.

A comprehensive study on the relationships of members of the *Actinobacteria* class has not been done prior to this work. 16S rDNA sequences from representative strains from each genus of *Actinobacteria* were used to construct a phylogenetic tree (**Figure 1**). It is important to note that, as an up-to-date authoritative list of validly described *Actinobacteria* is not available (e.g., *Bergey's Manual of Systematic Bacteriology*), orders, families, and genera that have been identified were taken from those available in the public database. Four subclasses were recognized: *Actinobacteridae, Acidimicrobidae, Coriobacteridae*, and *Rubrobacteridae*, with *Actinobacteridae* being the largest, consisting of 11 suborders and 43 families (**Table 2**). When generating the tree, care was taken to use sequences of good quality and the cutoff for the length of the sequence was 1400 bp. Any sequences below this were not included in the phylogenetic analysis. Sequences containing ambiguous bases were also excluded from the study. The phylogenetic tree was constructed with PHYLIP, the neighbor-joining method using the kimura-2-parameter model of sequence evolution. Bootstrap was used to calculate the confidence in the groupings of strains and the significant bootstrap values (>80) are indicated on the nodes (**Figure 1**).

The phylogenetic tree shows clear, distinct groupings of strains of particular genera in their respective families that were expected; however, some irregularities can be observed. The genus *Amycolatopsis* was proposed by Lechevalier and colleagues and, based on phylogenetic and chemotaxonomic properties, this genus is placed within the family Pseudonocardiaceae. The genus currently contains ten validated species that include *A. fastidiosa*. However, **Figure 1** clearly demonstrated *A. fastidiosa* to group with *Actinokineospora diospyrosa*, which belongs to the family Actinosynnemataceae, with a significant bootstrap value. In previous phylogenetic studies of the *Amycolatopsis* genus, *A. fastidiosa* consistently grouped outside other members of *Amycolatopsis*. In these studies only *Amycolatopsis* strains have been used occasionally alongside *Pseudonocardia*, but members of the closely related family Actinosynnemataceae were excluded. It is recommended that further work be done to establish to

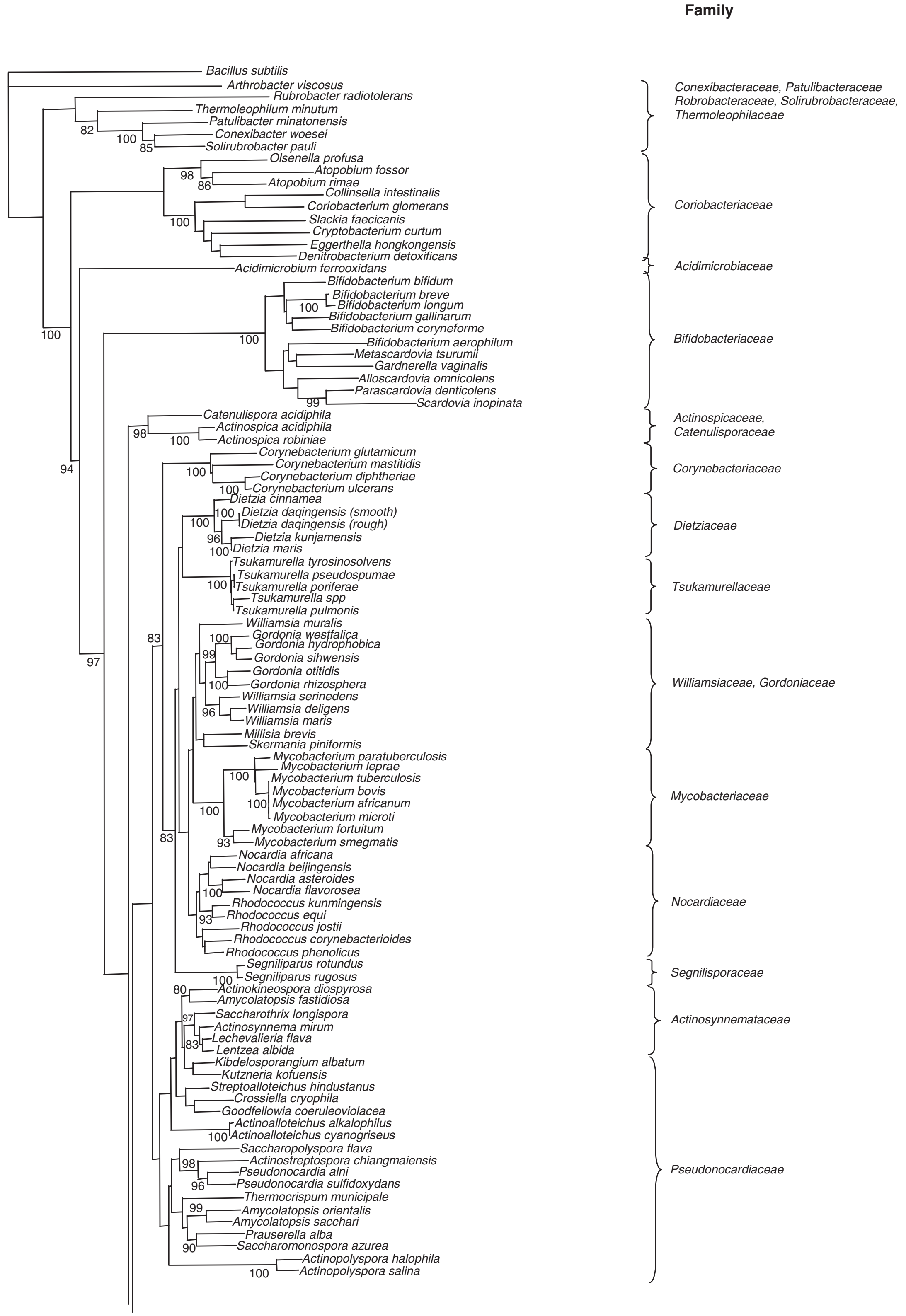
Family
Bacillus subtilis
Arthrobacter viscosus
Rubrobacter radiotolerans
Thermoleophilum minutum
Patulibacter minatonensis
Conexibacter woesei
Solirubrobacter pauli
82
100
85
Conexibacteraceae, Patulibacteraceae Robrobacteraceae, Solirubrobacteraceae, Thermoleophilaceae
Olsenella profusa
Atopobium fossor
Atopobium rimae
98
86
Collinsella intestinalis
Coriobacterium glomerans
Slackia faecicanis
Cryptobacterium curtum
Eggerthella hongkongensis
Denitrobacterium detoxificans
100
Coriobacteriaceae
Acidimicrobium ferrooxidans
Acidimicrobiaceae
Bifidobacterium bifidum
Bifidobacterium breve
Bifidobacterium longum
100
Bifidobacterium gallinarum
Bifidobacterium coryneforme
100
100
Bifidobacterium aerophilum
Metascardovia tsurumii
Gardnerella vaginalis
Alloscardovia omnicolens
Parascardovia denticolens
Scardovia inopinata
99
Bifidobacteriaceae
Catenulispora acidiphila
Actinospica acidiphila
Actinospica robiniae
98
100
94
Actinospicaceae, Catenulisporaceae
Corynebacterium glutamicum
Corynebacterium mastitidis
Corynebacterium diphtheriae
Corynebacterium ulcerans
100
100
Corynebacteriaceae
Dietzia cinnamea
Dietzia daqingensis (smooth)
Dietzia daqingensis (rough)
Dietzia kunjamensis
Dietzia maris
100
100
96
100
Dietziaceae
Tsukamurella tyrosinosolvens
Tsukamurella pseudospumae
Tsukamurella poriferae
Tsukamurella spp
Tsukamurella pulmonis
100
Tsukamurellaceae
Williamsia muralis
Gordonia westfalica
Gordonia hydrophobica
Gordonia sihwensis
Gordonia otitidis
Gordonia rhizosphera
Williamsia serinedens
Williamsia deligens
Williamsia maris
Millisia brevis
Skermania piniformis
83
97
100
99
100
96
Williamsiaceae, Gordoniaceae
Mycobacterium paratuberculosis
Mycobacterium leprae
Mycobacterium tuberculosis
Mycobacterium bovis
Mycobacterium africanum
Mycobacterium microti
Mycobacterium fortuitum
Mycobacterium smegmatis
100
100
100
93
83
Mycobacteriaceae
Nocardia africana
Nocardia beijingensis
Nocardia asteroides
Nocardia flavorosea
Rhodococcus kunmingensis
Rhodococcus equi
Rhodococcus jostii
Rhodococcus corynebacterioides
Rhodococcus phenolicus
100
93
Nocardiaceae
Segniliparus rotundus
Segniliparus rugosus
100
Segnilisporaceae
Actinokineospora diospyrosa
Amycolatopsis fastidiosa
80
Saccharothrix longispora
Actinosynnema mirum
Lechevalieria flava
Lentzea albida
97
83
Actinosynnemataceae
Kibdelosporangium albatum
Kutzneria kofuensis
Streptoalloteichus hindustanus
Crossiella cryophila
Goodfellowia coeruleoviolacea
Actinoalloteichus alkalophilus
Actinoalloteichus cyanogriseus
100
Saccharopolyspora flava
Actinostreptospora chiangmaiensis
Pseudonocardia alni
Pseudonocardia sulfidoxydans
98
96
Thermocrispum municipale
Amycolatopsis orientalis
Amycolatopsis sacchari
99
Prauserella alba
Saccharomonospora azurea
90
Actinopolyspora halophila
Actinopolyspora salina
100
Pseudonocardiaceae

Figure 1 (Continued)

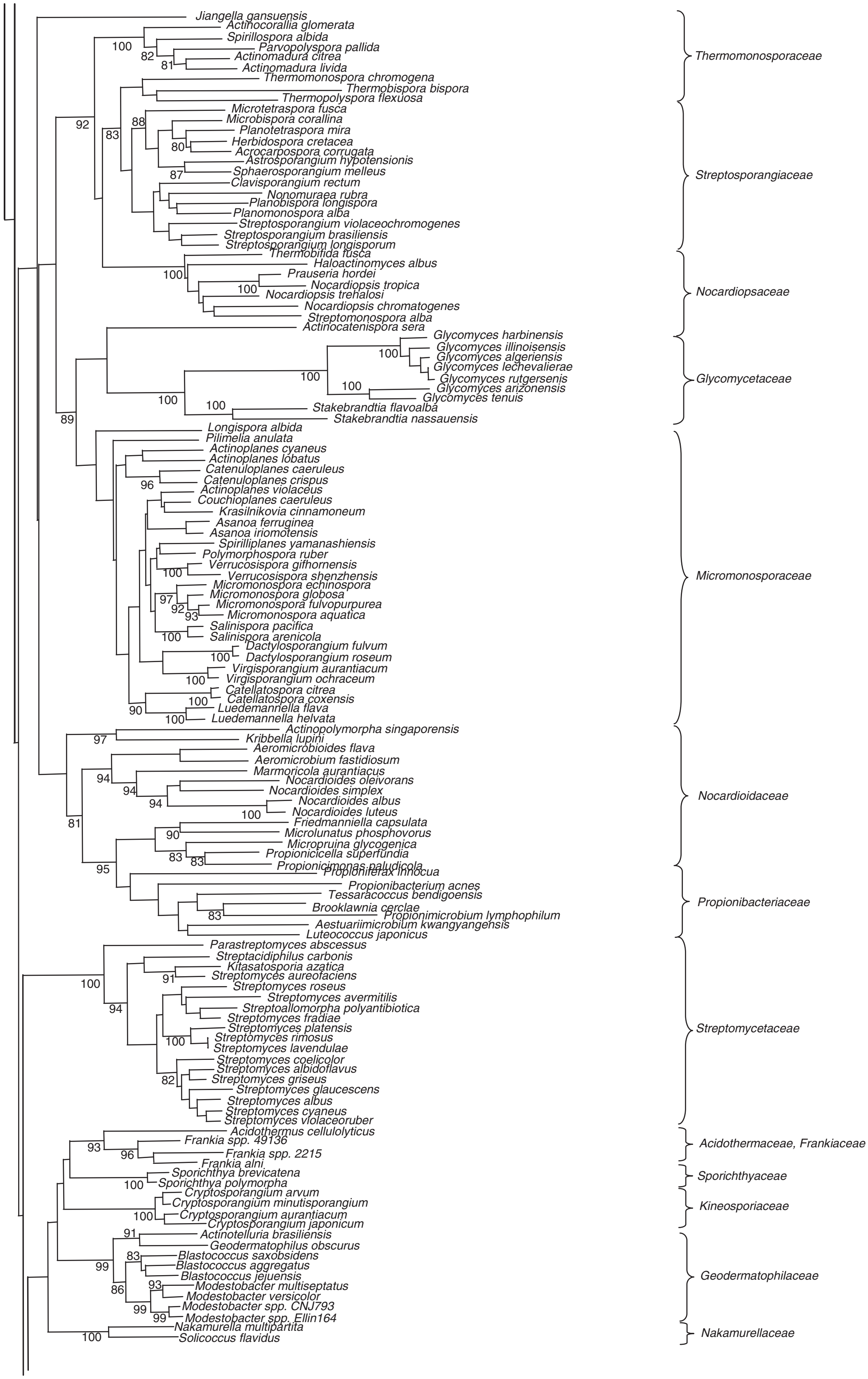
Jiangella gansuensis
Actinocorallia glomerata
Spirillospora albida
Parvopolyspora pallida
Actinomadura citrea
Actinomadura livida
Thermomonospora chromogena
Thermobispora bispora
Thermopolyspora flexuosa
Microtetraspora fusca
Microbispora corallina
Planotetraspora mira
Herbidospora cretacea
Acrocarpospora corrugata
Astrosporangium hypotensionis
Sphaerosporangium melleus
Clavisporangium rectum
Nonomuraea rubra
Planobispora longispora
Planomonospora alba
Streptosporangium violaceochromogenes
Streptosporangium brasiliensis
Streptosporangium longisporum
Thermobifida fusca
Haloactinomyces albus
Prauseria hordei
Nocardiopsis tropica
Nocardiopsis trehalosi
Nocardiopsis chromatogenes
Streptomonospora alba
Actinocatenispora sera
Glycomyces harbinensis
Glycomyces illinoisensis
Glycomyces algeriensis
Glycomyces lechevalierae
Glycomyces rutgersenis
Glycomyces arizonensis
Glycomyces tenuis
Stakebrandtia flavoalba
Stakebrandtia nassauensis
Longispora albida
Pilimelia anulata
Actinoplanes cyaneus
Actinoplanes lobatus
Catenuloplanes caeruleus
Catenuloplanes crispus
Actinoplanes violaceus
Couchioplanes caeruleus
Krasilnikovia cinnamoneum
Asanoa ferruginea
Asanoa iriomotensis
Spirilliplanes yamanashiensis
Polymorphospora ruber
Verrucosispora gifhornensis
Verrucosispora shenzhensis
Micromonospora echinospora
Micromonospora globosa
Micromonospora fulvopurpurea
Micromonospora aquatica
Salinispora pacifica
Salinispora arenicola
Dactylosporangium fulvum
Dactylosporangium roseum
Virgisporangium aurantiacum
Virgisporangium ochraceum
Catellatospora citrea
Catellatospora coxensis
Luedemannella flava
Luedemannella helvata
Actinopolymorpha singaporensis
Kribbella lupini
Aeromicrobioides flava
Aeromicrobium fastidiosum
Marmoricola aurantiacus
Nocardioides oleivorans
Nocardioides simplex
Nocardioides albus
Nocardioides luteus
Friedmanniella capsulata
Microlunatus phosphovorus
Micropruina glycogenica
Propionicicella superfundia
Propionicimonas paludicola
Propioniferax innocua
Propionibacterium acnes
Tessaracoccus bendigoensis
Brooklawnia cerclae
Propionimicrobium lymphophilum
Aestuariimicrobium kwangyangensis
Luteococcus japonicus
Parastreptomyces abscessus
Streptacidiphilus carbonis
Kitasatospora azatica
Streptomyces aureofaciens
Streptomyces roseus
Streptomyces avermitilis
Streptoallomorpha polyantibiotica
Streptomyces fradiae
Streptomyces platensis
Streptomyces rimosus
Streptomyces lavendulae
Streptomyces coelicolor
Streptomyces albidoflavus
Streptomyces griseus
Streptomyces glaucescens
Streptomyces albus
Streptomyces cyaneus
Streptomyces violaceoruber
Acidothermus cellulolyticus
Frankia spp. 49136
Frankia spp. 2215
Frankia alni
Sporichthya brevicatena
Sporichthya polymorpha
Cryptosporangium arvum
Cryptosporangium minutisporangium
Cryptosporangium aurantiacum
Cryptosporangium japonicum
Actinotelluria brasiliensis
Geodermatophilus obscurus
Blastococcus saxobsidens
Blastococcus aggregatus
Blastococcus jejuensis
Modestobacter multiseptatus
Modestobacter versicolor
Modestobacter spp. CNJ793
Modestobacter spp. Ellin164
Nakamurella multipartita
Solicoccus flavidus
Thermomonosporaceae
Streptosporangiaceae
Nocardiopsaceae
Glycomycetaceae
Micromonosporaceae
Nocardioidaceae
Propionibacteriaceae
Streptomycetaceae
Acidothermaceae, Frankiaceae
Sporichthyaceae
Kineosporiaceae
Geodermatophilaceae
Nakamurellaceae

Figure 1 (Continued)

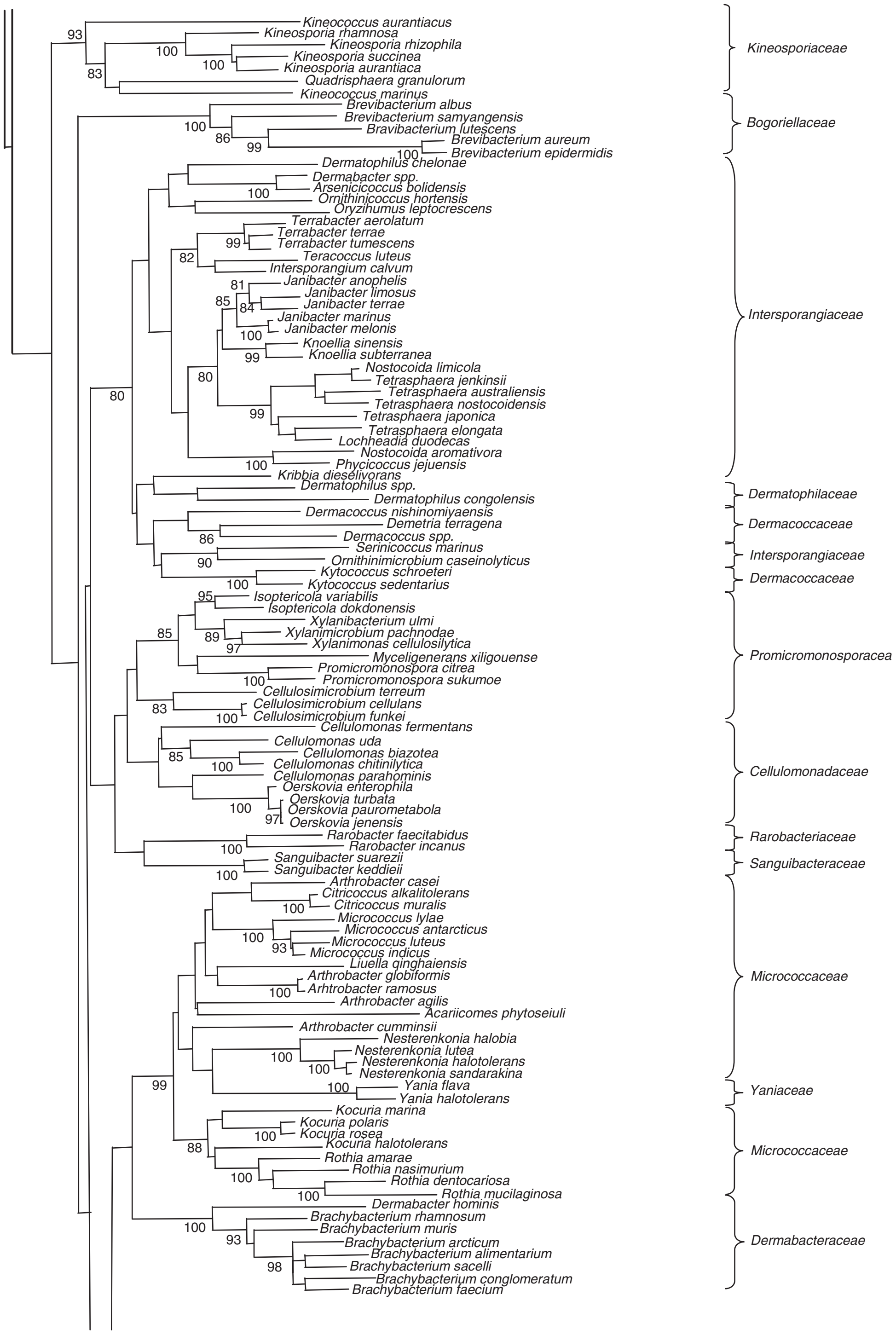
Kineococcus aurantiacus
93
Kineosporia rhamnosa
100
Kineosporia rhizophila
100
Kineosporia succinea
Kineosporia aurantiaca
83
Quadrisphaera granulorum
Kineococcus marinus
Kineosporiaceae
Brevibacterium albus
Brevibacterium samyangensis
100
Bravibacterium lutescens
86
Brevibacterium aureum
99
100
Brevibacterium epidermidis
Bogoriellaceae
Dermatophilus chelonae
Dermabacter spp.
Arsenicicoccus bolidensis
100
Ornithinicoccus hortensis
Oryzihumus leptocrescens
Terrabacter aerolatum
Terrabacter terrae
99
Terrabacter tumescens
82
Teracoccus luteus
Intersporangium calvum
81
Janibacter anophelis
Janibacter limosus
85
84
Janibacter terrae
Janibacter marinus
100
Janibacter melonis
Knoellia sinensis
99
Knoellia subterranea
Nostocoida limicola
80
Tetrasphaera jenkinsii
80
Tetrasphaera australiensis
Tetrasphaera nostocoidensis
99
Tetrasphaera japonica
Tetrasphaera elongata
Lochheadia duodecas
Nostocoida aromativora
100
Phycicoccus jejuensis
Intersporangiaceae
Kribbia dieselivorans
Dermatophilus spp.
Dermatophilus congolensis
Dermatophilaceae
Dermacoccus nishinomiyaensis
Demetria terragena
86
Dermacoccus spp.
Dermacoccaceae
Serinicoccus marinus
90
Ornithinimicrobium caseinolyticus
Intersporangiaceae
Kytococcus schroeteri
100
Kytococcus sedentarius
Dermacoccaceae
95
Isoptericola variabilis
Isoptericola dokdonensis
Xylanibacterium ulmi
85
89
Xylanimicrobium pachnodae
97
Xylanimonas cellulosilytica
Myceligenerans xiligouense
Promicromonospora citrea
100
Promicromonospora sukumoe
Promicromonosporacea
Cellulosimicrobium terreum
83
Cellulosimicrobium cellulans
100
Cellulosimicrobium funkei
Cellulomonas fermentans
Cellulomonas uda
85
Cellulomonas biazotea
100
Cellulomonas chitinilytica
Cellulomonas parahominis
Oerskovia enterophila
Oerskovia turbata
100
Oerskovia paurometabola
97
Oerskovia jenensis
Cellulomonadaceae
Rarobacter faecitabidus
100
Rarobacter incanus
Rarobacteriaceae
Sanguibacter suarezii
100
Sanguibacter keddieii
Sanguibacteraceae
Arthrobacter casei
Citricoccus alkalitolerans
100
Citricoccus muralis
Micrococcus lylae
Micrococcus antarcticus
100
Micrococcus luteus
93
Micrococcus indicus
Liuella qinghaiensis
Arthrobacter globiformis
100
Arhtrobacter ramosus
Arthrobacter agilis
Acariicomes phytoseiuli
Arthrobacter cumminsii
Nesterenkonia halobia
Nesterenkonia lutea
100
Nesterenkonia halotolerans
100
Nesterenkonia sandarakina
Micrococcaceae
99
100
Yania flava
Yania halotolerans
Yaniaceae
Kocuria marina
Kocuria polaris
100
Kocuria rosea
Kocuria halotolerans
88
Rothia amarae
Rothia nasimurium
100
Rothia dentocariosa
100
Rothia mucilaginosa
Micrococcaceae
Dermabacter hominis
Brachybacterium rhamnosum
100
Brachybacterium muris
93
Brachybacterium arcticum
Brachybacterium alimentarium
98
Brachybacterium sacelli
Brachybacterium conglomeratum
Brachybacterium faecium
Dermabacteraceae
80

Figure 1 (Continued)

which family *A. fastidiosa* belongs, and in future studies of *Amycolatopsis* strains members of Actinosynnemataceae should be included. The genus *Parvopolyspora* was described by Liu and Lian; however, since *Parvopolyspora pallida* has not been described efficiently according to the rules of Bacterial Nomenclature, it is not considered to be a valid genus/species. Using chemotaxonomic, morphological, physiological, and DNA–DNA hybridization methods, Itoh and colleagues and Miyadoh and colleagues showed *P. pallida* to be closely related to *Actinomadura* with the latter study proposing *P. pallida* to be transferred to the genus *Actinomadura.* Using 16S rRNA gene

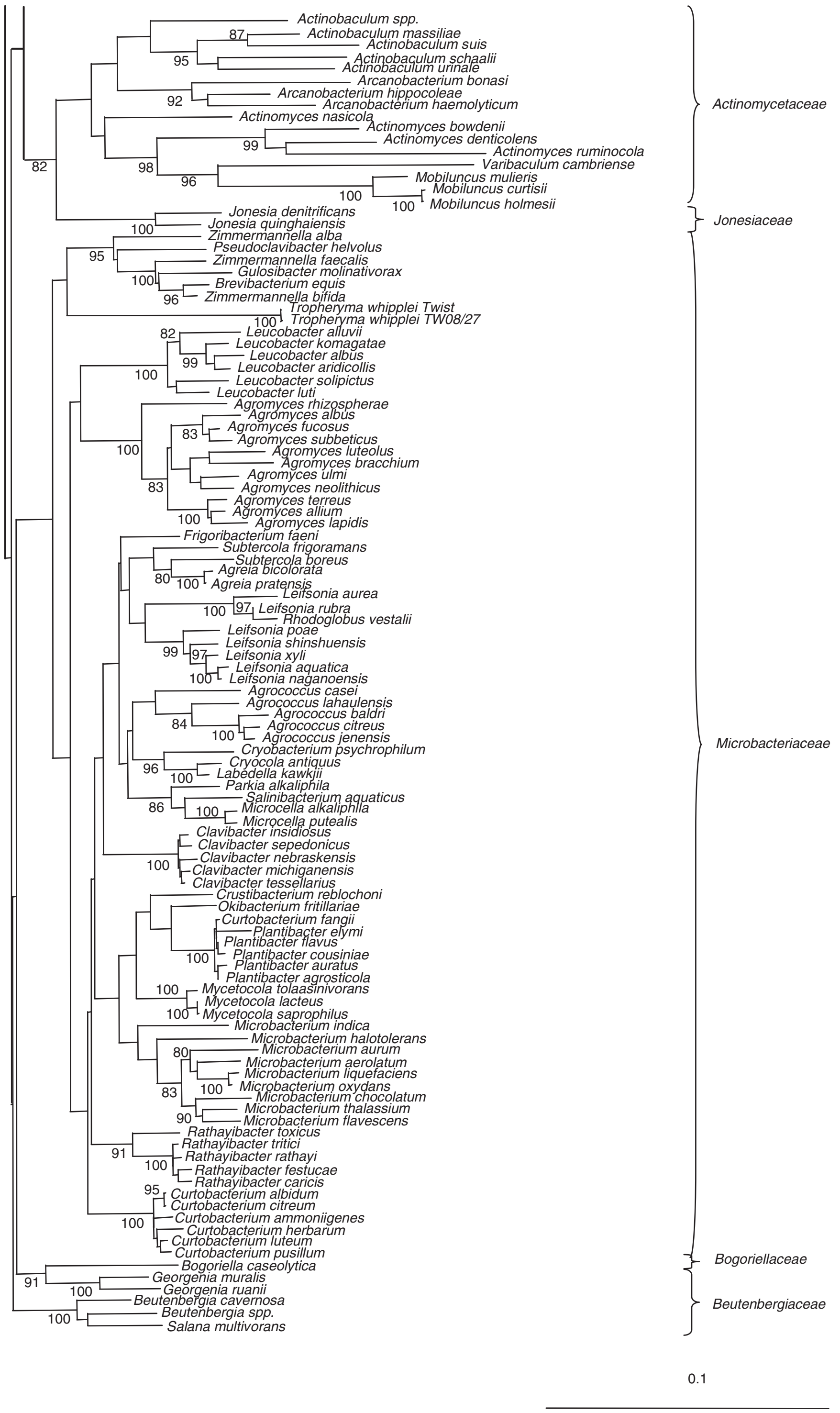
Actinobacterium spp.
87
Actinobaculum massiliae
Actinobaculum suis
95
Actinobaculum schaalii
Actinobaculum urinale
Arcanobacterium bonasi
Arcanobacterium hippocoleae
92
Arcanobacterium haemolyticum
Actinomyces nasicola
Actinomyces bowdenii
99
Actinomyces denticolens
Actinomyces ruminocola
82
98
Varibaculum cambriense
96
Mobiluncus mulieris
100
Mobiluncus curtisii
100
Mobiluncus holmesii
Actinomycetaceae
Jonesia denitrificans
100
Jonesia quinghaiensis
Jonesiaceae
Zimmermannella alba
95
Pseudoclavibacter helvolus
Zimmermannella faecalis
Gulosibacter molinativorax
100
Brevibacterium equis
96
Zimmermannella bifida
Tropheryma whipplei Twist
100
Tropheryma whipplei TW08/27
82
Leucobacter alluvii
Leucobacter komagatae
Leucobacter albus
99
Leucobacter aridicollis
100
Leucobacter solipictus
Leucobacter luti
Agromyces rhizospherae
Agromyces albus
83
Agromyces fucosus
Agromyces subbeticus
100
Agromyces luteolus
Agromyces bracchium
Agromyces ulmi
83
Agromyces neolithicus
Agromyces terreus
Agromyces allium
100
Agromyces lapidis
Frigoribacterium faeni
Subtercola frigoramans
Subtercola boreus
80
Agreia bicolorata
100
Agreia pratensis
Leifsonia aurea
100
97
Leifsonia rubra
Rhodoglobus vestalii
Leifsonia poae
Leifsonia shinshuensis
99
97
Leifsonia xyli
Leifsonia aquatica
100
Leifsonia naganoensis
Agrococcus casei
Agrococcus lahaulensis
84
Agrococcus baldri
Agrococcus citreus
100
Agrococcus jenensis
Cryobacterium psychrophilum
96
Cryocola antiquus
100
Labedella kawkjii
Parkia alkaliphila
86
Salinibacterium aquaticus
100
Microcella alkaliphila
Microcella putealis
Microbacteriaceae
Clavibacter insidiosus
Clavibacter sepedonicus
Clavibacter nebraskensis
100
Clavibacter michiganensis
Clavibacter tessellarius
Crustibacterium reblochoni
Okibacterium fritillariae
Curtobacterium fangii
Plantibacter elymi
Plantibacter flavus
100
Plantibacter cousiniae
Plantibacter auratus
Plantibacter agrosticola
100
Mycetocola tolaasinivorans
Mycetocola lacteus
100
Mycetocola saprophilus
Microbacterium indica
Microbacterium halotolerans
80
Microbacterium aurum
Microbacterium aerolatum
Microbacterium liquefaciens
83
100
Microbacterium oxydans
Microbacterium chocolatum
Microbacterium thalassium
90
Microbacterium flavescens
Rathayibacter toxicus
91
Rathayibacter tritici
100
Rathayibacter rathayi
Rathayibacter festucae
Rathayibacter caricis
95
Curtobacterium albidum
Curtobacterium citreum
Curtobacterium ammoniigenes
100
Curtobacterium herbarum
Curtobacterium luteum
Curtobacterium pusillum
Bogoriella caseolytica
Bogoriellaceae
91
Georgenia muralis
100
Georgenia ruanii
Beutenbergia cavernosa
Beutenbergia spp.
100
Salana multivorans
Beutenbergiaceae
0.1

Figure 1 Phylogenetic analysis of species belonging to the class *Actinobacteria* using the near entire 16S rDNA gene sequences. The phylogenetic tree was construced using the neighbour-joining method and Kimura-2-parameter of sequence evolution. Numbers on the branches indicate the percentage bootstrap value of 100 replicates. The scale bar indicates 10% nucleotide dissimilarity (10% nucleotide substitutions per 100 nucleotides).

sequences, Tamura and Hatano confirmed previous work that favored the transfer to *Actinomadura*. In the current study, *P. pallida* groups with *Actinomadura* strains, further confirming earlier work on *P. pallida*, and calls for an emended description of the genus *Actinomadura* to be made to accommodate *P. pallida* as *Actinomadura pallida*.

Table 2 Members comprising the class *Actinobacteria*

Class	*Subclass*	*Order*	*Suborder*	*Family*
Actinobacteria	Acidimicrobidae	Acidimicrobiales	Acidimicrobineae	Acidimicrobiaceae (1)
	Actinobacteridae	Actinomycetales	Actinomycineae	Actinomyceaceae (5)
			Catenulisporineae	Actinospicaceae (1)
				Catenulisporaceae (1)
			Corynebacterineae	Corynebacteriaceae (1)
				Dieziaceae (1)
				Gordoniaceae (3)
				Mycobacteriaceae (1)
				Nocardiaceae (3)
				Segniliparaceae (1)
				Tsukamurellaceae (1)
				Williamsiaceae (1)
			Frankineae	Acidothermaceae (1)
				Frankiaceae (1)
				Geodermatophilaceae (4)
				Kineosporiaceae (3)
				Nakamurellaceae (1)
				Sporichthyaceae (1)
			Glycomycineae	Glycomycetaceae (2)
			Micrococcineae	Beutenbergiaceae (3)
				Bogoriellaceae (1)
				Brevibacteriaceae (1)
				Cellulomonadaceae (3)
				Dermabacteraceae (2)
				Dermacoccaceae (3)
				Dermatophilaceae (2)
				Intrasporangiaceae (14)
				Jonesiaceae (1)
				Microbacteriaceae (24)
				Micrococcaceae (10)
				Promicromonosporaceae (7)
				Rarobacteraceae (1)
				Sanguibacteraceae (1)
				Yaniaceae (1)
			Micromonosporineae	Micromonosporaceae (18)
			Propionibacterineae	Nocardioidaceae (11)
				Propionibacteriaceae (8)
			Pseudonocardineae	Actinosynnemaaceae (6)
				Pseudonocardiaceae (17)
			Streptomycineae	Streptomycetaceae (8)
			Streptosporangineae	Nocardiopsaceae (4)
				Streptosporangiaceae (13)
				Thermomonosporaceae (4)
		Bifidobacteriales		Bifidobacteriaceae (3)
	Coriobacteridae	Coriobacteriales	Coriobacterineae	Coriobacteriaceae (8)
	Rubrobacteridae	Rubrobacterales	Rubrobacterineae	Conexibacteraceae (1)
				Paulibacteraceae (1)
				Rubrobacteraceae (1)
				Solirubrobacteraceae (1)
				Thermoleophilaceae (1)

Reproduced from sequences available from NCBI. Numbers in parenthesis indicate the number of genera in each family.

Microbispora bispora is currently recognized as a member of the Pseudonocardiaceae family, yet in **Figure 1** *M. bispora* is next to *Thermopolyspora flexuosa* within the family Streptosporangiaceae, distinctly separate from Pseudonocardiaceae. Grouping outside both *M. bispora* and *T. flexuosa* is *Thermomonospora chromogena*, which belongs to the family Thermomonosporaceae. These three strains form a separate group from the Pseudonocardiaceae, Thermomonosporaceae, and Streptosporangiaceae families, being closer to the last. The three strains also share many chemotaxonomic traits, so re-examination of these strains is advised to determine their exact positions within the three families. *Prauseria hordei* is also recognized as a member of Pseudonocardiaceae, though this strain has not been validly described in the literature. Analysis of its 16S rRNA gene has placed it among the Nocardiopsaceae to a strain of *Nocardiopsis* with a bootstrap value of 100, with the 16S rDNA sequences of the two strains showing 99% sequence similarity.

The phylogenetic position of *T. whipplei* has been under some considerable debate. This has largely been due to the lack of chemotaxonomic studies as this organism has been difficult to culture. Initially, *T. whipplei* was placed as a deep-branching strain of the Cellulomonadaceae family. Only recently, La Scola and colleagues gave a detailed description of the Whipple's disease bacillus with regard to its cultivation and morphology. The new genus and species name of *T. whipplei* was based solely on the 16S rRNA gene sequence data as it was not possible to study any chemotaxonomic traits. In a phylogenetic tree presented in *The Practical Streptomyces Handbook*, *T. whipplei* grouped closer to Microbacteriaceae, although not placed within it. Chater and Chandra constructed a phylogenetic tree using the 16S rRNA genes of sequenced *Actinobacterial* genomes, which included two strains of *T. whipplei.* In this study, *T. whipplei* grouped with *Leifsonia* and also showed a close relationship to *Streptomyces* strains. **Figure 1** demonstrates that *T. whipplei* is a member of the Microbacteriaceae family, as indicated by Chater and Chandra, but is not as close to the *Streptomyces* genus as shown in their tree.

Attention is also drawn to the families Dermabacteraceae, Intrasporangiaceae, Dermacoccaceae, and Dermatophilaceae. The last accommodates two genera, *Dermatophilus* and *Kineosphaera.* The genera *Dermacoccus, Kytococcus*, and *Demetria* make up the family Dermacoccaceae and 14 genera have been described for Intrasporangiaceae with Dermabacteraceae containing two genera, *Brachybacterium* and *Dermabacter*. It is evident from **Figure 1** that some strains from these families have been misclassified. For example, a *Dermabacter* species groups with a member of Intrasporangiaceae, *Arsenicicoccus bolidensis*, with a high bootstrap value. The classification of members of these families, in particular Dermatophilaceae and Dermacoccaceae, needs to be studied again to clarify their taxonomic positions.

Speciation of Genera Using Protein-Coding Genes

A number of studies have made use of other housekeeping genes to support the phylogeny derived from the 16S rRNA gene. The genes chosen for phylogenetic analysis must fulfill certain criteria in that they must be essential and distributed among the genera, therefore reducing the possibility of HGT. They must also have an evolutionary rate higher than the 16S rRNA gene, thus providing better resolution of closely related strains. Examples of the housekeeping genes that have been used in conjunction with the 16S rRNA gene include *recA, gyrB, trpB, rpoB, secA1, hsp65, sodA*, and *trpB*. In many of these studies, the use of alternative genes resolved the relationships between closely related species. Ul-Hassan examined the *S. violaceoruber* cluster and phylogenetic analysis was done using 16S rRNA, *gyrB, recA*, and *trpB* genes. The strains formed a tight monophyletic cluster in the partial 16S rRNA gene tree. The results of the *gyrB, recA*, and *trpB* analysis correlated with the 16S rRNA analysis as the topology of the trees and grouping of the strains were identical. Phylogenetic histories for the housekeeping genes were generated and the relative separation in phylogenetic tree space was examined using the Robinson–Foulds distance metric. This confirmed that the genes *gyrB, trpB*, and *recA* show a faster evolutionary rate than 16S rRNA, and therefore being good choices for use alongside the 16S rRNA gene. Analysis of the *S. violaceoruber* strains with *recA, gyrB*, and *trpB* showed no further resolution of the intrageneric relationships between the closely related species. These results were in agreement with those of Duangmal and colleagues, who used the entire 16S rRNA sequence to study type members of the *S. violaceoruber* cluster, as well as soil isolates, and showed that members of the *S. violaceoruber* cluster are highly homogeneous.

The study of Ul-Hassan was similar to a multilocus sequence typing (MLST) approach. MLST is a method for the genotypic characterization between closely related species using the allelic mismatch of a number of housekeeping genes (usually seven). This is a powerful tool which has been used in molecular epidemiology for phylogenetic analysis of bacterial pathogens. *Bifidobacterium* strains have been shown to have high levels of sequence similarity of the 16S rDNA gene ranging from 87.7 to 99.5%, with some strains possessing identical sequences, thus making it difficult to identify and characterize strains. Ventura and colleagues developed MLST to study strains of the *Bifidobacteria* genus. Analysis using the 16S rRNA sequences allowed the discrimination of most species within the genus, but it was more difficult to do so between subspecies. For MLST analysis the genes *clpC, dnaB, dnaG, dnaJi, purF, rpoC*, and *xlp* were used. The phylogenetic tree generated from the concatenated sequences showed a significant increase in the discriminatory power between the strains.

Genome Structure and Evolution

The linearity of the streptomycete chromosome was first determined in *S. lividans* and has subsequently been seen in other members of the genera. Redenbach and colleagues set out to analyze whether large linear chromosomes were a distinct feature of *Streptomyces* species. Linearity of the chromosomes was determined by PFGE. The results of this study concluded that members of the genera which undergo a complex cycle of morphological differentiation (e.g., *Streptomyces, Micromonospora, Actinoplanes*, and *Nocardia*) possess large linear chromosomes, whereas actinobacterial strains with simpler life cycle (*Mycobacterium, Corynebacterium*, and *Rhodococcus*) have smaller, circular chromosomes. Genomes of 19 medically and industrially important *Actinobacteria* have been completely sequenced and annotated. Analyses of whole genome sequences have provided an insight into how different *Actinobacteria* have become adapted to their particular ecological niches.

The *Streptomyces* Genome

Of the streptomycetes, complete genomes of *S. coelicolor* A3(2) and *S. avermitilis* are available. *S. coelicolor* A3(2) is genetically the best-known representative of the genus as nearly all major achievements in streptomycete genetics and physiology have been done in this model organism. *S. avermitilis* is an important organism in the pharmaceutical industry as it is a major producer of avermectins, which are antiparasitic agents used in human and veterinary medicine. Both *S. coelicolor* A3(2) and *S. avermitilis* have linear chromosomes of 8.7 and 9 Mb respectively. *Streptomyces* species are the predominant *Actinobacteria* in soil, which is a highly heterogeneous matrix composed of organic, inorganic, and gaseous material. Organisms must withstand extremes of temperature and moisture, particularly in the upper layers of the soil profile. Streptomycetes have a saprophytic lifestyle and their ability to successfully colonize the soil is due to the production of a variety of extracellular hydrolytic enzymes. These include nucleases, lipases, amylases, xylanases, proteinases, and chitinases, thus making streptomycetes central organisms in decomposition.

Analysis of the types and location of the genes in the *S. coelicolor* A3(2) chromosome suggests that it comprises a central core region and a pair of chromosomal arms. Genes with an essential function such as in DNA replication, transcription, and translation are located in the core region whereas those with a nonessential function (e.g., secondary metabolism) are located in the arm regions. Significant synteny between the core of the *S. coelicolor* A3(2) genome and the whole genomes of *M. tuberculosis* and *C. diphtheriae* was observed, suggesting these to have a common ancestor, whereas the arms of the *S. coelicolor* A3(2) chromosome consisted of acquired DNA.

Genetic instability of the Streptomyces *genome*

The extreme variability of *Actinobacteria* is a well-known phenomenon first demonstrated by the work of Lieske, and is clearly evident when examining culture plates. In nearly all cases, genetic instability has a pleiotropic effect and can result in the loss of antibiotic biosynthesis and resistance, pigment production, and aerial mycelium formation. Genes can be lost in various frequencies from 10^{-4} to 10^{-2} per spore; these deletions can remove up to 25% of the genome. For a chromosome of 8 Mb this can be up to 2 Mb, which exceeds the size of a small bacterial genome. Initially, genetic instability was considered to be a consequence of the linear structure of the chromosome. However artificially circularized, chromosomes were found to be more unstable than the parent chromosome and it was only after the deletion of the terminal regions that the circular chromosomes became stable structures. Lin and Chen (1997) proposed the high numbers of transposable elements (TEs) in the terminal regions to be responsible for genetic instability. Approximately 40% of TEs are located in the terminal regions of the *S. coelicolor* A3(2) genome, with a similar pattern being seen in *S. avermitilis*. TEs can be found in multiple copies in the genome and this can often lead to DNA rearrangements in the form of transpositions, insertions, deletions, and gene transfer events. The structure of the *Streptomyces* chromosome and the distribution of the essential and nonessential genes and TEs provides some benefit to the organism in that it allows a certain amount of plasticity to the genome. As the terminal regions contain only a few essential genes they are more tolerant to DNA rearrangements and acquisitions. Using DNA microarrays, 14 regions were identified in the *S. coelicolor* A3(2) genome that were absent in its close relatives and of these regions, 11 were located in the arms of the chromosome. Prior to sequencing of the *S. coelicolor* A3(2) genome, gene clusters for actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, and the *whiE* cluster had been analyzed. Sequencing of the chromosome revealed ~20 more clusters encoding putative secondary metabolites. These include clusters for coelichelin, coelibactin, geosmins, desferrioxamines, and hopanoids (**Table 3**).

Reductive Genomes

The ability to easily acquire and lose DNA without causing detrimental effects to the organisms plays a major role in the evolution of these free-living saprophytic bacteria. In contrast to this, obligate intracellular pathogens occupy a stable environmental niche, so gene transfer does not play such a crucial role in the evolution and adaptation of these organisms. A process of reductive evolution is seen to occur in these pathogens where a number of gene functions become redundant as the host will supply these needs. The presence of pseudogenes in a genome gives an estimation of gene decay. A gene for a particular function will be made redundant when the functional constraint is relaxed, thus making it prone to inactivating mutations. When these mutations become fixed in a population the gene becomes a

Table 3 Secondary metabolites produced by *S. coelicolor* A3(2)

Secondary metabolites	*Location on chromosome*
Known structures	
Antibiotics	
Actinorhodin	SCO5071-SCO5092
Calcium dependant antibiotic	SCO3210-SCO3249
Prodiginines	SCO5877-SCO5898
Methylenomycin	SCP1 plasmid
Siderophores	
Coelibactin	SCO7681-SCO7691
Coelichelin	SCO0489-SCO0499
Desferrioxamines	SCO2782-SCO2785
Pigments	
Isorenieratene	SCO0185-SCO0191
Tetrahydroxynaphthalene	SCO1206-SCO1208
TW95a (*whiE*) spore pigment	SCO5314-SCO5320
Lipids	
Eicosapentaenoic acid	SCO0124-SCO0129
Hopanoids	SCO6759-SCO6771
Other molecules	
Butyrolactones	SCO6266
Geosmin	SCO6073
Unknown structure	
Chalcone synthases	SCO7669-SCO7671, SCO7222
Deoxysugar synthases/ glycosyl transferases	SCO0381-SCO0401
Nonribosomal peptide synthetases	SCO6429-SCO6438
Sesquiterpene cyclase	SCO5222-SCO5223
Siderophore synthetase	SCO5799-SCOSCO5801
Type I polyketide synthases	SCO6273-SCO6288, SCO6826-SCO6827
Type II fatty acid synthase	SCO1265-SCO1273

Taken from Bentley *et al*. (2002) and Challis and Hopwood (2003).

pseudogene. These genes will either remain in the genome and be subjected to further mutations to such an extent that they are no longer recognizable or are completely removed.

M. leprae

The best documented example of reductive evolution is seen in *M. leprae*. The complete genomes of *M. leprae* and *M. tuberculosis* have been sequenced and are much smaller than the streptomycete genomes: 3.2 Mb (*M. leprae*) and 4.4 Mb (*M. tuberculosis*). When the two mycobacterial strains are compared they show a large difference in GC content with *M. leprae* having an average GC of 57.8% while that of *M. tuberculosis* is 65.6%. The most striking feature of the *M. leprae* genome is that it contains 49.5% protein-coding genes (1604 genes) compared to 90.8% (3959) protein-coding genes in *M. tuberculosis*. The number of pseudogenes in *M. leprae* is 1116, with only six being found in *M. tuberculosis*, indicating massive gene decay in *M. leprae*. It is now proposed that *M. leprae* has evolved to have the natural minimal gene set for Mycobacteria and, unlike *M. tuberculosis*, has a limited metabolic repertoire and host range. Reduction in the genome size is also connected with the observation that intracellular pathogens make extensive use of host cellular processes.

T. whipplei

The genome of *T. whipplei* is the most extreme example of an Actinobacterium that has undergone genome reduction. *T. whipplei* is the causative agent of Whipples disease, which is characterized by malabsorption and is a systemic infection affecting any part of the body. Like *M. leprae, T. whipplei* was also difficult to culture and it was only in 2000 that it was grown in human fibroblasts and exhibits a slow doubling time of 17 days comparable to 14 days for *M. leprae*. The genome sequence of the *T. whipplei* Twist strain has recently become available and shows it to have a small circular chromosome of only 0.92 Mb. The average GC content of the genome is 46%, which is considerably lower than that of streptomycetes and mycobacterial strains and is in contrast to the reduced *M. leprae*. Analysis of the genome has shown that enzymes involved in information processing (DNA/RNA polymerases and gyrases) are present. In *M. leprae*, genes for biosynthesis of amino acids were present, suggesting that these are limiting in their environment, but in *T. whipplei* it became apparent that complete and partial losses of some amino acid biosynthesis gene clusters have occurred. This implies that amino acids are obtained from the host. At least two amino acids and peptide ABC transport systems were identified in the genome of *T. whipplei*. An interesting observation from the genome included the identification of *whiA* and *whiB*, which in *S. coelicolor* are involved in sporulation. Spores have not been reported in *T. whipplei* under laboratory conditions, although they may perhaps arise in the environment. The genome shows little evidence of gene acquisition, which is a common property of reduced genomes. In other examples of reduced genomes like those of *M. leprae* and *Rickettsia prowazekii*, high numbers of pseudogenes are present, suggesting that these genomes are still in the process of downsizing. In contrast, the *T. whipplei* genome contains a few pseudogenes, indicating that no further genome decay is occurring.

P. acnes

P. acnes is a commensal bacterium found on human skin with preference to sebaceous follicles, but it can be an opportunistic pathogen and can cause acne. *P. acnes* contains a single circular chromosome of 2.5 Mb encoding 2333 genes. Putative functions were assigned to 68% of the genes with 20% sharing no significant similarity to any database entries. The presence of 35 pseudogenes containing frameshift mutations or premature stop codons leads the author to suggest this gene decay to be a recent event. Analysis of the whole genome led to ten regions being identified as possibly being of foreign origin, one of which contained genes for the biosynthesis of lanthionine. Other genes identified were involved in substrate uptake and pathogenicity. With regard to the physiology of *P. acnes*, all genes of the Embden–Meyerhof and pentose-phosphate pathway are present. *P. acnes* can grow anaerobically on a number of substrates, and enzymes required for this have been identified.

Prior to the genome being sequenced, GehA was recognized as an extracellular triacylglycerol lipase, which degrades skin tissue components. The ability of lipases to degrade human skin lipids results in the production of fatty acids that assist in bacterial adhesion and colonization of the follicles. Many other lipases and esterases have been identified, including endoglycoceramidase that breaks down glycosphingolipids, which exist in the cell membranes of all vertebrates. Three putative sialidase enzymes have been identified, along with sialic acid transport proteins, suggesting that *P. acnes* cleaves sialoglycoconjugants to obtain sialic acid as a source of carbon and energy. Homologs of CAMP factors have also been recognized in *P. acnes* and these had only previously been detected in streptococcal species. CAMP factors are secreted proteins that are known as pathogenic determinants and have lethal effects when given to mice and rabbits. CAMP factors have been suggested to act as pore-forming toxins. Three enzymes with putative hemolytic activities show some resemblance to hemolysin III of *Bacillus cereus*. Many surface proteins that can act as antigens have been identified and can trigger an inflammatory response that is seen during acne.

An interesting feature of some of the genes is the presence of continuous stretches of 12–16 guanine or cytosine residues, either in the promoter region or at the 5′end. The length of this poly(C)/(G) tract is variable and is generated during replication by slipped-strand mispairing. These tracts are involved in phase variation, which serves as an adaptation mechanism whereby the organism can change its phenotype to evade immune responses or rapidly adapt to environmental changes.

Bifidobacteria longum

Members of the *Bifidobacterium* genus comprise 3–6% of the adult fecal flora and the presence of these organisms is thought to provide health benefits. This has led to the increase in the use of *Bifidobacterium* species in health-promoting foods. *Bifidobacterium* species are obligate anaerobes and the majority of strains have been isolated from mammalian gastrointestinal tracts (GITs). They are among the first to colonize GITs of newborn babies until weaning, when *Bacteroides* take over. This system of successive colonization is thought to play a major role in the build-up of immune system tolerance. Thus a complex balance of microflora is needed for a normal and healthy digestive system. This becomes evident after antimicrobial therapy, where the incidence of GIT disorders greatly increases. However, little is known about the physiology and genetics of the organisms and the mechanism of host-microbe interaction. Sequencing the genome of *B. longum* has provided important clues into the adaptation of *Bifidobacterium* to GITs of humans. The genome of *B. longum* is 2.3 Mb and it is estimated that 86% of the genome is protein coding. No aerobic or anaerobic respiratory components were identified, confirming *B. longum* to be a strict fermentative anaerobe. Homologs of the enzymes needed for the fermentation of glucose, including the fructose-6-phosphate shunt and a partial Embden–Meyerhof pathway, are present. Enzymes that are needed to feed many sugars into the fructose-6-phosphate are present, further confirming the ability of *B. longum* to ferment a large variety of sugars.

B. longum is able to ferment amino acids through the use of 2-hydroxyacid and other predicted deaminases and dehydratases. More than 20 putative peptidases have been predicted and these enable *B. longum* to obtain amino acids from proteinaceous material in the GIT where carbohydrates are less abundant. *Bifidobacterium* species colonize the lower GIT, which tends to be poor in mono- and disaccharides as they are taken up by the host and the microflora in the upper GIT. As a result, more that 8.5% of the predicted proteins are dedicated to carbohydrate transport and metabolism. Many glycosyl hydrolases have been predicted in the genome and these cover a wide range of substrates including di-, tri-, and higher order oligosaccharides. Oligosaccharide transporters have also been identified, which may aid *B. longum* to compete for the uptake of structurally diverse oligosaccharides. Unlike most bacteria, *B. longum* makes great use of negative transcriptional control to regulate gene expression, with nearly 70% of its transcriptional regulators being repressors. Negative repressors are thought to allow for a more precise response to changes in the environment, which is consistent with the need of *B. longum* to adapt to the constantly changing conditions of the GIT.

Industrially Important Phenotypes of *Actinobacteria*

The best-studied example of an important industrial application of *Actinobacteria* is the production of antibiotics by streptomycete strains. As mentioned previously, the discovery of actinomycin and streptomycin in the early 1940s led many large pharmaceutical companies in different parts of the world to initiate large screening programs in a hope of finding novel antibiotic compounds. This resulted in a rapid increase in the rate at the discovery of new compounds between the 1940s and the 1960s. These years are now considered to be the Golden Age of antibiotic discovery, as after 1960s the rate at which new compounds were discovered decreased sharply. Initial methods used in isolating new compounds were based on simple plating procedures where the soil samples were mixed with water and subsequent filtering to remove large soil particles. The extract was then plated on nutrient medium. Representative colonies were isolated and studied further for antibiotic production. It is now well known that antibiotic production is part of secondary metabolism and occurs only under certain nutritional conditions. One method of discovering new compounds is by isolating new organisms, and Takahashi and Ōmura provide an excellent review on different isolation methods that have been developed based on this rationale.

Considerable interest has been applied in screening marine organisms for the discovery of novel compounds. The study of Okazaki and colleagues and subsequent research by the group has reported the isolation of an actinobacterial strain from the Sagami bay area producing a novel bioactive compound. This isolate would only produce this compound in selective sea water containing Japanese seaweed. This study highlighted the fact that normal culture media are not sufficient for these organisms as they have adapted to producing bioactive compounds under marine-specific nutritional conditions. It is therefore important to study and understand the physiology of marine *Actinobacteria* to develop effective techniques for their isolation. *Actinobacteria*-specific bacteriophage have been successfully used for isolation and identification of novel or rare actinobacterial strains from terrestrial environments and to determine the relatedness of actinobacterial strains. Kurtboke (2005) developed an improved method for detecting marine *Actinobacteria.* This method uses the actinophage to reduce the number of common marine organisms that tend to outgrow any rare *Actinobacteria*, increasing the likelihood of isolating new actinobacterial strains that potentially produce novel bioactive compounds. Actinophage can be used for host identification at the genus and the species level. In general, streptomycetes phage are genus-specific, although some cross-reactivity has also been detected with other genera, including *Nocardia, Streptosporangium*, and *Mycobacterium.*

Sequencing of mycobacterial strains is done mainly because they are important human and animal pathogens, but the genomes of two nonpathogenic mycobacterial strains have been sequenced. *Mycobacterium* sp. KMS was isolated from soil sites that had been polluted by creosols, pentachlorophenol, and polycyclic aromatic hydrocarbons (PAHs), which contain up to four aromatic rings. Another strain of *Mycobacterium vanbaalenii* PYR-1 was found to possess the remarkable ability to degrade PAHs, including alkyl- and nitro-substituted PAHs such as naphthalene. Both these mycobacterial species use dioxygenases and monooxygenases for the oxidation of the ring component of these compounds. PAH compounds are toxic and have carcinogenic properties, and microbial degradation of these compounds is the most effective method of remediation of the contaminated soil. Vinyl chloride is a potential carcinogen and tends to accumulate as an end-product of dechlorination of solvents such as perchloroethylene and trichloroethene.

A strain of *Nocardioides* sp. JS614 was isolated from an industrial soil site that was contaminated with vinyl chloride and 1,2-dichloroethane. Higher growth yields were obtained when *Nocardioides* sp. JS614 was grown on media containing vinyl chloride than without; the strain is unusually sensitive to vinyl chloride starvation. This strain possess a 300-kb plasmid carrying genes encoding monooxygenases and epoxyalkane: coenzyme M transferase, thought to be involved in degradation of vinyl chloride. *Nocardioides* species are known to degrade other aromatic compounds, including 2,4,6-trinitrophenol, phenanthrene, and dibenzofuran (DF). Carbazole is an N-heterocyclic aromatic compound derived from creosote and crude and shade oil and is known to be both toxic and mutagenic. It is widely used as a raw material for the production of dyes, medicines, and plastics. *Nocardioides aromaticivorans* IC177 is able to degrade carbazole. Inoue and colleagues were able to clone and partially sequence the *car* genes responsible for carbazole degradation. The sequences showed similarities to genes found in *Pseudomonas* and *Sphingamonas* strains.

Arsenic is a highly toxic metal and its presence in the environment is largely from a geochemical source (rocks and minerals), although anthropogenic action has led to its increase. Many organisms have been documented to be able to transform arsenic, through either reduction or oxidation reactions; these include *Cenibacterium arsenoxidans, Alcaligenes faecalis, Agrobacterium tumefaciens, Bacillus*, and *Shewanella.* An arsenic-defence mechanism is present in all organisms studied for arsenic degradation. Members of the genus *Corynebacterium* are of great biotechnological importance, especially for the large-scale production of amino acids such as L-glutamate and L-lysine. Members of the coryneform bacteria (*C. glutamicum* and *C. lactofermentum*) are resistant to arsenic and genes involved in this process are contained in two operons, *ars1* and *ars2.* Research of Mateos and colleagues aims to make use of genetically engineered strains of *C. glutamicum* for bioremediation of arsenic from heavily contaminated water sites.

Members of the genus *Gordonia* belong to the same family as Corynebacteria (Corynebacteriaceae). A strain was isolated from polluted water taken from the inside of a deteriorated rubber tyre. By using chemotaxonomy, DNA–DNA hybridization and 16S sequence analysis this strain was found to represent a new species within the genus *Gordonia* and was given the name *Gordonia westfalica.* It could utilize natural and synthetic components of rubber, including *cis*-1,4-polyisoprene. *Janibacter terrae* strain XJ-1 was found to have the ability to degrade DF. Polychlorinated dibenzo-*p*-dioxins (PCDOs) and polychlorinated DF (PCDFs) are common pollutants in the environment and are released as contaminants in pesticides and herbicides. These are highly toxic compounds and tend to accumulate in the body fat of animals. A DF-degrading strain of *J. terrae,* which can use DF as a sole source of carbon and energy, was also isolated. *J. terrae* contains the *dbdA* (DF dioxygenase) gene cluster and sequence analysis demonstrated it to be nearly identical to the cluster found on a large plasmid of *Terrabacter* sp. YK3, which utilizes DF in a similar manner.

Among the *Actinobacteria, Rhodococcus* species are well-known for their ability to biodegrade and transform a wide range of complex organic compounds. It is for this reason they have been referred to as 'masters of catabolic versatility' by Larkin and colleagues. Many of the genes required for degradation of xenobiotic compounds are encoded on plasmids, including those for polychlorinated biphenyls, isopropylbenzene, and indene. Genes associated with virulence in pathogenic strains (*R. equis*) are also encoded on plasmids. Readers are recommended the articles by Larkin and colleagues, Sekine and colleagues, and Gurtler and colleagues for the detailed analysis of the overall metabolic diversity and genetics of these organisms. Pesticides are composed of compounds with varying chemical structures, including organochlorides, *s*-trazines, triazinones, organophosphates, and sulfonylureas. Members of *Actinobacteria* play a major role in biotransformation and biodegradation of these chemicals, where a single strain can degrade more than one compound though co metabolism. De Schrijver and De Mot provide a comprehensive review on the degradation of pesticides by *Actinobacteria.*

It is clear from the examples discussed above that members of the *Actinobacteria* play a major role in bioremediation and biodegradation of complex xenobiotic compounds in the environment. They have the genetic capabilities to either utilize these compounds as sources of energy or break them down to simpler forms, which in turn can be used by other organisms (cometabolism). *Actinobacteria* also have an enormous biotechnological potential. As mentioned before members of the *Actinobacteria*, in particular the genus *Streptomyces*, are major producers of medically important antibiotics. Examples of other uses of Actinobacteria are listed in **Table 4**.

Table 4 Uses of actinobacterial strains in biotechnology

Organism	*Biotechnological uses*
Mycobacterium(nonmedical strains)	• Biotransformation of steroids • Removal of vinyl chloride from industrial waste • Production of optically active epoxides which are subsequently used for chemical synthesis of optically active pharmaceutical compounds
Corynebacterium (nonmedical strains)	• *C. glutamicum* used for the large-scale production of L-glutamic acid and L-lysine. These strains can also be modified to produce threonine, isoleucine, tylosine, phenylalanine, and tryptophan • Fermentative production of nucleotides which are used as flavor enhancers in foods
Microbispora rosea	• Produces D-xylose isomerase, which converts glucose into fructose, which is subsequently used to produce high-fructose syrup
Micrococcus species	• Used for processing of fermented meats to improve color, aroma, flavor and keeping quality • Synthesis of long-chain aliphatic hydrocarbons that have the potential to be processed into lubricating oils or other petroleum substitutes
Rhodococcus species	• Rhodococcal nitrile converting enzymes used to convert nitriles into their corresponding higher value acids and amides, which can be used as polymers in dispersants, flocculants, and superabsorbents • Commercial production of biosurfactants. • Capacity to degrade a diverse range of hydrocarbons, including halogenated and long chain as well as aromatic compounds • Biopurification of coal and crude oil by removing contaminating organosulfur compounds
Frankia	• Along with other actinorhizal organisms are used where it is necessary to rapidly establish a plant cover
Cellulomonas species	• Used for single-cell protein production from a variety of waste products • Mixed cultures can be used to convert xylan into methane via hydrolysis, acidogensis, and methanogensis • Photoevolution of molecular hydrogen using cellulose as sole carbon source
Micromonospora	• Commercial scale production of amylases and cellulases • Vitamin B_{12} production
Brevibacterium	• Used for cheese ripening
Nocardioides	• Used for their ability to perform chemical and enzymatic modifications of complex compounds and production of industrially important enzymes

Concluding Remarks

The application of chemotaxonomy, numerical taxonomy, and DNA–DNA hybridization methods have provided a basis for studying the taxonomy of *Actinobacteria.* New advances in DNA technology have contributed considerably to bacterial taxonomy, in particular sequence analysis of small subunit rRNA. Sequence analysis of the 16S rRNA is routinely used in conjunction with analysis of chemotaxonomic traits to identify and describe existing and newly isolated strains. Phylogenetic analyses of families comprising the *Actinobacteria* class have been studied in great detail with regard to the genera they accommodate; however, in many of these studies members of other closely related families are not included. The aim of this chapter was to examine the phylogenetic relationships of all the genera that have been described as *Actinobacteria* using the entire 16S rDNA gene sequences available in the public database. The results of this study correlated well with previous analyses, and clusters that have already been defined by numerical taxonomy were retained. This study also highlights the importance of using a combination of traditional methods along with newer molecular based techniques for taxonomic purposes. Also when generating the phylogeny of strains, the analysis of more strains from closely related families is essential to allow a more accurate discrimination between strains.

Bacterial genomes are under constant selection pressure whether they are in the environment in the presence of toxic chemicals, living a saprophytic lifestyle in the soil, or as obligate intracellular pathogens. *Actinobacteria* represent a heterogeneous group of organisms that have the ability to adapt to their particular ecological niches. Free-living species such as *Streptomyces* have made use of HGT, tolerating acquisition and loss of genes, thus allowing them to acclimatize to their fluctuating environment more rapidly. In contrast, pathogenic bacteria (*Mycobacterium* and *Tropheryma*) have chosen the path of reductive evolution where nearly all genes not essential for growth are lost. *Actinobacteria* represent an important group of organisms for the bioremediation of water and soil sites that have been polluted with toxic recalcitrant compounds. The increasing availability of whole genome sequences of actinobacterial strains and their ongoing analysis has revealed the enormous genetic capabilities of this important group of bacteria.

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Relevant Website

http://www.genomesonline.org – GOLD Genomes OnLine Database v 2.0

Adhesion, Microbial

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Defining Statement
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Biological Significance of Microbial Adhesion
Mechanisms of Microbial Adhesion
Selected Survey of Specific Adhesion Strategies
Consequences of Microbial Adhesion in Human Disease
Targeting Adhesion to Inhibit Bacterial Virulence
Further Reading

Glossary

biofilm A community of microorganisms associated with a surface.

microbial ecology The study of interactions and relationships between microorganisms and their environment.

planktonic cells Cells grown predominantly in suspension as individual cells in a liquid medium.

teichoic acids A class of negatively charged polymers expressed on the cell surface of Gram-positive bacteria that are either linked covalently to the peptidoglycan (wall teichoic acids) or linked to lipids in the cytoplasmic membrane (lipoteichoic acids).

Abbreviations

CF	cystic fibrosis
DAF	decay-accelerating factor
ECM	extracellular matrix
GbO4	globotetraosylceramide
IBCs	intracellular bacterial communities
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection
Tafi	thin aggregative fimbriae

Defining Statement

Microbial adhesion is crucial to the survival and lifestyle of many microorganisms. Both beneficial and pathogenic relationships forged between microbe and host depend on adhesive events and colonization. This article highlights the highly evolved microbial adhesion mechanisms and discusses the prevalence and implications of adhesion in diverse ecosystems.

Introduction

From the center of the earth and deep-sea vents to plant roots and the human intestine, microorganisms occupy remarkably diverse niches on our planet. These microbes include bacteria, archaea, fungi, and protista, and are found attached to rocks and soil particles, corals, and ocean sponges. Bacteria, for example, symbiotically colonize plants and humans as well as fish and squid, resulting in mutual benefit to both microbe and host. Pathogenic and unwelcome bacteria can egress from their native niche and adhere to and infect other sites and host tissues, leading to cellular injury and disease. Microbes also adhere to the hulls of ships and to machinery in food-processing factories, resulting in contamination and adverse circumstances. Specific adhesion strategies have evolved in order to facilitate microbial attachment to diverse substrata in both symbiotic and pathogenic associations. Understanding the molecular mechanisms and functional implications of microbial adhesion is crucial for generating complete descriptions of our ecosystems, understanding and predicting ecosystem stability due to globalization and climate change, and attempting to control and prevent the unfortunate and often devastating consequences of infectious diseases. Thus, microbial adhesion is a fundamental component of the field of microbial ecology. This article will focus on the adhesive strategies employed specifically by bacteria, though many parallels can be found in the arsenal of adhesive strategies harbored by the other classes of microbes. We will highlight several exciting and up-to-date scientific discoveries as a platform to illustrate the biological significance and implications of microbial adhesion.

Biological Significance of Microbial Adhesion

The propensity for bacteria to associate with surfaces (living or abiotic) in nearly all ecosystems far exceeds the tendency to persist in suspension, living freely in a planktonic state. Attachment to surfaces allows bacteria to persist in advantageous locations where there may be high nutrient concentrations or to provide protection from hostile environments. In numerous instances, bacteria form biofilms – structurally complex and dynamic bacterial communities. The metabolic labor of acquiring nutrients is divided, sometimes according to spatial coordinates in the community, and distribution is promoted through an organized architecture of community members. Protection from harsh environmental conditions is a major benefit of life in a biofilm and the first line of defense is provided by members residing at the edges of the community. Under certain conditions, bacteria disperse from the biofilm, to seek a new environment and potentially readhere and colonize new niches. Adhesion events are crucial to biofilm formation, growth, and development. We set the stage for discussing the mechanisms of microbial adhesion by first illustrating a few examples across a broad landscape in which bacterial adhesion (often followed by biofilm formation) takes place.

Adhesion in the Water

The coral reefs are home to an enormous diversity of marine life, including beautiful fish, mollusks and urchins, and the significantly smaller microorganisms with which they cohabit. Bacteria are, in fact, an integral constituent of the microbiota of healthy corals. They colonize distinct sites in coral tissue including the surface mucous layer and porous components in the coral skeleton where they fix nitrogen, decompose chitin, and provide organic compounds. The molecular mechanisms of adhesion and the sustained interactions between bacteria and their coral hosts are currently not well understood but are key questions being addressed in the emerging field of coral microbiology. Understanding the microbial interactions that promote health versus those that cause disease is important in efforts to preserve and prevent further destruction of coral reefs worldwide.

Adhesion of bacteria in many water environments inevitably has detrimental consequences. Bacterial adhesion and biofilm formation on the hulls of ships creates resistance to water flow, increases drag, and thus decreases the efficiency of movement through the water. Microbial fouling is an economic and environmental burden in this way and in several industrial settings including drinking water pipes and oil pipelines, where the proliferation of sulfide-producing bacteria leads to the deterioration and corrosion of the steel surfaces. Understanding the mechanisms of adhesion is key to developing strategies to control and prevent these adverse and costly consequences.

Adhesion to Plants

Rhizobia are Gram-negative soil bacteria that adhere to and colonize the root cells of leguminous plants, including soybeans and alfalfa. Upon entry into a root hair, rhizobia traverse a distance to the center of the root hair cell and together with proliferating plant cells form a nodule. Here, rhizobia fix nitrogen, converting molecular nitrogen (N_2) from the air into ammonia, nitrates, and other nitrogenous compounds to support plant metabolism. Rhizobia are particularly important to plants in nitrogen-deficient soils. In return, rhizobia receive carbon-rich organic compounds, important for their own energy production, from the plant.

Other beneficial symbionts include *Bacillus thuringiensis*. This bacterium is an important Gram-positive pathogen whose insecticidal properties have gained attention in the development of crops genetically modified to express the bacterium’s potent toxin, now referred to as Bt transgenic crops. In the wild, *B. thuringiensis* colonizes the surface of some plants and exists naturally in some caterpillars. The bacterium produces a unique kind of endotoxin, a proteinaceous crystal that is lethal to several pests, including flies, mosquitoes, and beetles, upon ingestion. This symbiosis with plants is dependent on initial host–microbe adhesion events.

The attractive chemical signals and ultimate adhesive interactions of *Agrobacterium tumefaciens* with wounded plants leads to the unfortunate development of tumors on the lower stems and main roots, the hallmark of Crown Gall diseases. Attachment is the first step in the pathogenic cascade and takes place in the soil around the roots – the rhizosphere. In a two-step adhesive process, initial weak binding interactions are followed by the bacterial expression of multiple gene products to synthesize cellulose and anchor the microbe to the host tissue, while enhancing adhesive interactions between bacteria in the microcolony. Adhesive plant proteins called vitronectins are also implicated in the adhesion process. Subsequent DNA transfer and integration of a specific fragment of DNA (the transfer DNA) from the bacterium to a plant cell results in the expression of several oncogenic genes and the formation of tumors.

Adhesion in the Human Host

The normal and healthy human body is composed of approximately ten times more bacterial cells than human cells. These bacteria comprise our microbiota and are colonized in distinct sites throughout the body, including the skin and mouth, and the small intestine and colon. In the mouth and small intestine, bacterial adhesion is critical to maintenance of microbial populations, where either salivary flow or movement of contents eliminates the nonadherent bacteria. Our microbiota is, in general, beneficial. Bacteria in the gut, for example, attach to undigested by-products and degrade some polysaccharides into carbon and energy sources, for example. Recent results indicate that the balance of bacterial populations in the gut influence caloric intake through complex interbacterial metabolic networks and further study may help to understand and potentially control (decrease or increase) caloric uptake. Indeed, the microbiota is dynamic and shifts in balance that alter the sizes of different bacterial populations can also lead to proliferation of disease-causing opportunistic pathogens. In addition, the inoculation of the human host with bacteria from the environment is a common source of infectious disease, particularly in the hospital setting. The consequences of bacterial adhesion in human infectious diseases are numerous and will be addressed in more detail after the description of specific adherence mechanisms.

Mechanisms of Microbial Adhesion

General Physicochemical Factors Affecting Adhesion

Mechanisms of microbial attachment are incredibly diverse and can be generally classified as either general nonspecific interactions or specific molecular-recognition binding events that involve the presentation of specific adhesive proteins on the bacterial cell surface. Of course, multiple mechanisms can act cooperatively to promote adhesion. A successful adhesive event depends on properties of both the bacterium and the substratum. Nonspecific interactions are the primary form of attachment to abiotic surfaces in aquatic and soil environments. Van der Waals interactions are attractive, usually weak, noncovalent forces that can operate at large separation distances (>50 nm) between the bacterium and the surface. At smaller distances (10–20 nm), electrostatic interactions participate and compete as attractive and repulsive forces. The net surface charge of most bacteria is negative due to cell wall and cell membrane components including negatively charged phosphate groups, carboxyls, and other acidic groups, in addition to surface-exposed proteins. Thus, bacteria like to adhere to positively charged surfaces. Typical binding surfaces, however, have a net negative surface charge, creating electrostatic repulsion that must be overcome by other physicochemical factors. The entire binding process is akin to a tug-of-war. The ionic strength of the surrounding medium affects the electrostatic interactions, and the aforementioned repulsion is eliminated, for example, in most aquatic environments due to high ionic strength resulting from high salt concentration. In the range of near contact (0.5–2 nm), hydrophobic interactions are important for bacterial adhesion. Energetically, the association of nonpolar groups on a bacterial surface with hydrophobic surfaces compensates for the unfavorable displacement of water molecules at that surface. When separated by less than 1 nm, stronger interactions including hydrogen bonding and the formation of salt bridges contribute to surface adhesion.

Specific Adhesin–Receptor Mechanisms

On many biotic surfaces, the adhesive forces and interactions described above promote the formation of an initial interface, but require concomitant or subsequent specific adhesive interactions to enable firm adhesion. Adhesin is the term ascribed to the surface-exposed bacterial molecule that mediates specific binding to a receptor or ligand on a target cell. It is not unusual for bacteria to harbor several types of specific adhesive machinery to provide adhesive capacity to multiple receptor molecules or to permit adhesion under changing environmental conditions such as temperature, pH, or nutrient status, where one adhesive strategy may be more effective than another.

Bacteria can produce a diverse array of adhesins with varying specificities for a wide range of host receptor molecules. Adhesion mechanisms can be classified according to the type of adhesin–receptor pair. Many bacterial adhesins function as lectins and the interactions between bacterial lectins and host cell carbohydrates are among the best-characterized attachment processes. Hallmark examples of carbohydrate recognition include *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* adhesion in the respiratory tract, *Escherichia coli* adhesion in the urinary tract and intestine, and *Helicobacter pylori* adhesion in the stomach. Other adhesins recognize specific amino acid-recognition motifs in proteins expressed on host cell surfaces. Extracellular matrix (ECM) proteins that are not directly integrated into the host cell also serve as attractive binding platforms for many bacteria, and numerous adhesins bind to these components in order to indirectly hijack the host signaling pathways, often to enable host cell internalization. Another general category of adhesins includes nonproteinaceous molecules such as lipopolysaccharides and teichoic acids, synthesized by Gram-negative and Gram-positive bacteria, respectively.

Most adhesins are incorporated into heteropolymeric extracellular fibers called pili or fimbriae. Bacteria invest enormous cellular resources to assemble fimbriae in order to present adhesins at the right time and the right place to initiate attachment when conditions are favorable and to permit detachment when necessary. Indeed, hundreds of such fibers have been described in Gram-negative organisms, and although they have diverse functions, many appear critical to binding, invasion, and survival of pathogenic microorganisms in the human host. Four distinct assembly mechanisms have emerged as the most well studied and include the chaperone–usher pathway, the general secretion pathway, the extracellular nucleation–precipitation pathway, and the alternate chaperone pathway. Gram-positive pathogens also produce adhesive pili. Unlike their Gram-negative counterparts, Gram-positive pili are formed by covalent polymerization of pilin subunits. A representative set of fimbrial adhesins is provided in **Table 1**.

Some bacteria present afimbrial adhesins on their surface. These are expressed as monomeric proteins or protein complexes that assemble at the cell surface and recognize host cell surface elements. Adhesins of the Dr family are expressed by *E. coli* strains and mediate recognition of decay-accelerating factor (DAF). DAF is found in the respiratory, urinary, genital, and digestive tracts, and Dr-mediated adhesion is important for binding in the intestine and urinary tract. Adhesive autotransporters represent a class of afimbrial adhesins expressed by a variety of unrelated microorganisms, including species of *Rickettsia*, *Bordetella*, *Neisseria*, *Helicobacter*, and many members of the family Enterobacteriaceae. *H. influenzae*, a causative agent of sinusitis, bronchitis, otitis media, and pneumonia, expresses an adhesive autotransporter termed Hap. Hap mediates binding to laminin, fibronectin, and collagen, all components of the ECM.

The most comprehensive descriptions of bacterial adhesion have emerged from studies of pathogenic bacteria involved in infectious diseases. Examples of these host–microbe interactions as well as some involved in the attachment of bacteria to plants, either as symbionts or as pathogens, are described in more detail below to highlight the remarkable diversity, specificity, and complexity among microbial adhesive strategies.

Selected Survey of Specific Adhesion Strategies

Pilus-Mediated Adhesion to Carbohydrates in the Urinary Tract

Uropathogenic *E. coli* (UPEC) colonize the gut as well as the genitourinary tract and produce numerous important adhesins and adhesive organelles to mediate adhesion in these niches. For example, FimH and PapG adhesins are presented at the tips of type 1 and P pili, respectively. FimH-presenting type 1 pili are required for *E. coli* to cause cystitis, or infection of the bladder, and PapG-presenting P pili are associated with pyelonephritis, infection of the kidney. Type 1 and P pili are composite heteropolymeric structures, with a distal tip fibrillum joined to a thicker rigid helical rod and both are assembled by the chaperone–usher system. More than 100 chaperone–usher systems have been identified through comparative genome analyses and many are

Table 1 Representative fimbrial adhesins and disease association

Organism(s)	*Adhesin*	*Assembly proteins*	*Associated fiber*	*Associated disease(s)*
Escherichia coli	FimH	FimC/FimD	Type 1 pili	Cystitis
	PapG	PapD/PapC	P pili	Cystitis/ pyelonephritis
	PrsG	PrsD/PrsC	Prs pili	Cystitis
	SfaS	SfaE/SfaF	S pili	UTI, newborn meningitis
	CooD	CooB/CooC	CS1 pili	Diarrhea
	CsgA	CsgB (nucleator), CsgE/CsgF (assembly), CsgG (secretion)	Curli	Sepsis
Salmonella typhimurium		PefD/PefC	Pef pili	Gastroenteritis
		LpfB/LpfC	Long polar fimbriae	Gastroenteritis
Salmonella enteritidis	AgfA	AgfB (nucleator)	Sef17 (thin aggregative fimbriae)	
Klebsiella pneumoniae	MrkD	MrkB/MrkC	MR/K (type 3) pili	Pneumonia
Bordetella pertussis	FimD	FimB/FimC	Type 2 and 3 pili	Whooping cough
Yersinia enterocolitica		MyfB/MyfC	Myf fimbriae	Enterocolitis
Neisseria gonorrhoea	PilC	General secretion apparatus	Type 4 pili	Gonorrhea
Pseudomonas aeruginosa, *Vibrio cholerae*, *Mycobacterium bovis*	Pilin protein			Cholera
Haemophilus influenzae		HifB/HifC	Hif pilus	Otitis media, meningitis

well studied and required for the assembly of extracellular adhesive organelles in pathogens including *Salmonella*, *Haemophilus*, *Klebsiella*, and *Yersinia*. In each chaperone–usher system, pilus assembly requires a unique protein pair (a chaperone and a usher) to facilitate the folding, transport, and ordered assembly of pilus subunits at the cell surface. This process begins in the periplasm, after subunit expression and translocation by the general secretory pathway into the periplasm. Periplasmic pilus chaperones consist of two immunoglobulin (Ig)-like domains and bind to folded subunits to keep their interactive surface capped and prevent nonproductive subunit aggregation. Pilin subunits also have an Ig-like fold, but they lack the seventh β strand, thus exposing the hydrophobic core. In a process termed donor strand complementation, the chaperone's G_1 β strand serves as the pilin's seventh strand, catalyzing the folding of the subunit. Chaperone–subunit complexes are targeted to an outer membrane usher to facilitate chaperone uncapping, translocation of subunits across the outer membrane, and pilus assembly. This occurs via a process termed donor strand exchange, in which the G_1 β strand of the chaperone is replaced by an N-terminal extension of the next pilus subunit. Thus in the mature pilus, each subunit incorporates its neighbor's N-terminal extension as part of its own Ig fold. Subunits have distinct specificity for other interactive subunits, such as the adhesin, and this confers distinct roles in pilus adhesion, initiation, elongation, termination and regulation.

The FimH adhesin, incorporated at the tip of the type 1 pilus, consists of a pilin subunit and the receptor-binding domain (**Figure 1**). The primary carbohydrate specificity of FimH is mannose. Interestingly, different *E. coli* isolates present FimH variants (specific allelic variations in protein sequence and structure) that exhibit varying specificities for monomannose and trimannose binding. FimH expressed by most commensal isolates of the intestine exhibit a higher specificity for trimannose-presenting glycoprotein receptors, whereas urinary tract isolates encode for a FimH variant with higher affinity for monomannose. In the latter, FimH mediates adhesion to the monomannose-containing glycoprotein uroplakin Ia that is expressed on the surface of superficial facet cells – the epithelial cells that line the lumen bladder (**Figure 1**).

Presented at the tips of P pili, the PapG adhesin mediates binding to a different carbohydrate receptor, the α-D-galactopyranosyl-(1–4)-β-D-galactopyranoside moiety of glycolipids presented by cells predominantly in the kidney. PapG variants (G-I, G-II, and G-III) exhibit altered specificities for three Galα(1–4)Gal-containing isoreceptors: globotriaosylceramide, globotetraosylceramide (GbO4), and globopentaosylceramide (the Forssman antigen). The demonstrated allelic variation in PapG and FimH binding specificities supports the notion that,

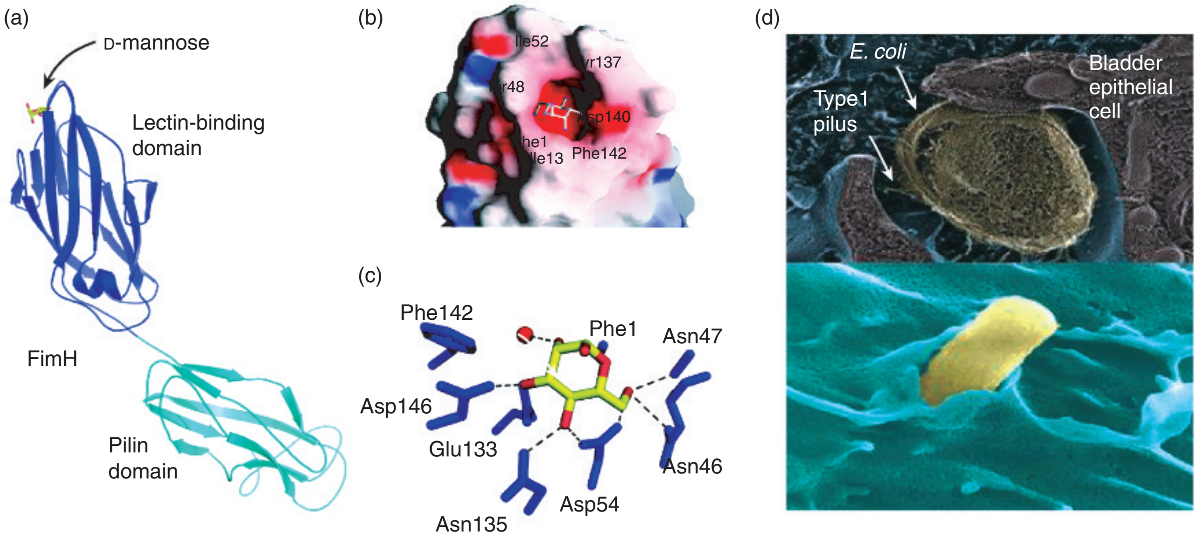

Figure 1 The FimH adhesin and type 1 pili-mediated adhesion of *E.coli*. (a) The ribbon representation of FimH (from the crystal structure of the FimCH complex). D-mannose is located at the top of the molecule. (b) Molecular surface representation in which the electrostatic potential surface with positively charged residues is shown in blue, negatively charged residues in red, and neutral and hydrophobic residues in white. Residues defining the hydrophobic ridge around the mannose-binding pocket are labeled. (c) The mannose-binding site with FimH residues. Mannose residues are shown with carbon atoms in yellow, oxygen atoms in red, and nitrogen atoms in blue. (d) Type 1 pili-mediated attachment of uropathogenic *E. coli* (UPEC) to the luminal surface of the bladder epithelium. (Top) High-resolution, freeze-fracture, deep-etch electron micrograph is from Mulvey MA, Lopez-Boado YS, Wilson CL, *et al*. (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *E. coli*. *Science* 282: 1494–1497. Reprinted with permission from AAAS. (Bottom) Scanning electron micrograph of a bacterium entering the membrane of bladder epithelial cells is reprinted from Soto GE and Hultgren SJ (1999) Bacterial adhesins: Common themes and variations in architecture and assembly. *Journal of Bacteriology* 181: 1059–1071.

through bacterial evolution, pathoadaptive mutations are selected for increasing the fitness of pathogenic organisms in distinct niches in the host.

Adhesion to ECM Components

The ECM contains a diverse array of oligosaccharides, proteoglycans, and proteins and functions to provide structural support and adhesive interactions among cells. Prevalent components include collagen, fibronectin, laminin, and vitronectin, as well as molecules such as heparan sulfate and chondroitin sulfate. Fibronectin is present in most tissues and fluids of the body and helps to create a cross-linked network between cells by presenting binding sites for other ECM components, a process that pathogenic organisms exploit to gain a foothold in host tissue. The ability to adhere to ECM components is a primary adhesion mechanism that contributes to the virulence of many pathogenic microorganisms. *Staphylococcus aureus* is a significant cause of nosocomial and often persistent infections. Among other ECM-binding proteins, *S. aureus* expresses the fibronectin-binding proteins FnBP-A and FnBP-B that permit adherence to fibronectin that are bridged to cellular integrins. This crucial binding event leads to host cell cytoskeletal rearrangements and invasion. *Streptococcus pyogenes* is armed with more than 12 fibronectin- and collagen-binding proteins. Like the FnBP-A adhesin in *S. aureus*, the major *S. pyogenes* adhesin, SfbI, and the *Yersinia* adhesin, YadA, bind to fibronectin and bridge the bacteria to integrins, leading to integrin clustering and eventual internalization. Invasin is a *Yersinia* adhesin that bypasses the ECM and binds directly to integrin transmembrane receptors. Other less-ubiquitous ECM components also serve as binding receptors for bacterial adhesins and their sites of expression often relate to the tissue tropism of a particular bacterial pathogen.

Curli-Mediated Multipurpose Adhesion

Curli are a unique class of adhesive extracellular amyloid fibers produced by Gram-negative bacteria, including *E. coli*. The highly homologous fibers produced by *Salmonella* species are called Tafi (thin aggregative fimbriae). The fibers mediate biofilm formation and attachment to host proteins including fibronectin, laminin, and plasminogen, and have been implicated in human sepsis. When expressed together with cellulose, curli and Tafi contribute to a remarkable aggregative phenotype characterized by a patterned assembly of cells radiating from the center when grown on a surface such as agar. Curli are assembled by the nucleation–precipitation pathway, and assembly requires specific molecular machinery encoded by the *csgBA* and *csgDEFG* operons (**Figure 2**). The major subunit protein (CsgA) and the nucleator

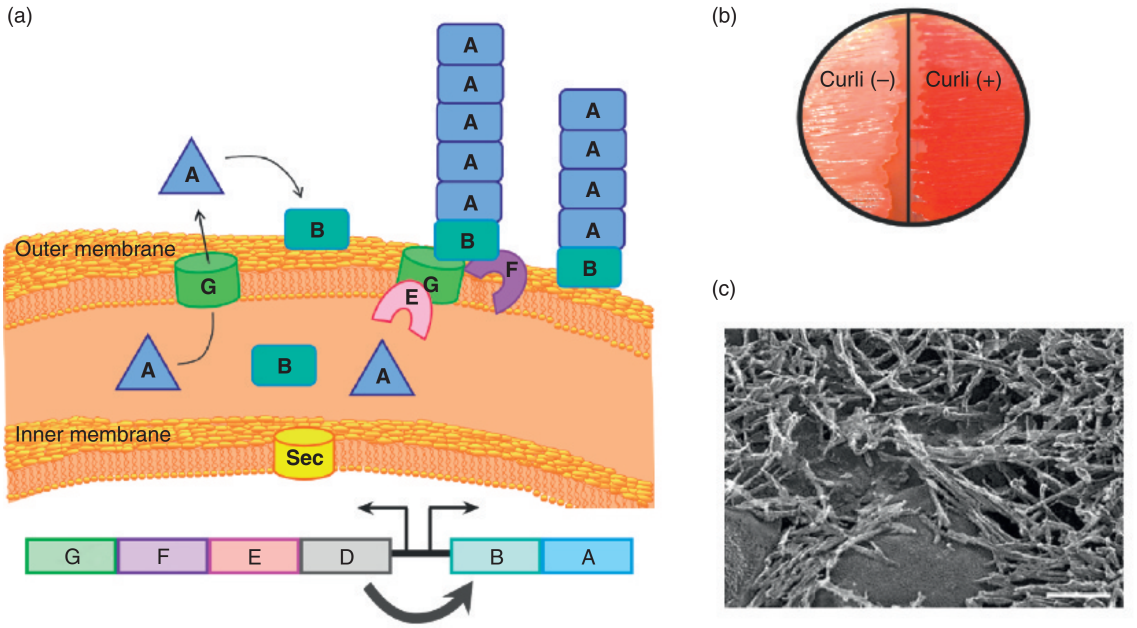

Figure 2 Curli biogenesis and biology. (a) Current model of curli biogenesis. Curli assemble through the nucleation–precipitation pathway. Polymerization of the major curli subunit protein, CsgA into β-sheet-rich amyloid fibers depends on the nucleating activity of the minor subunit, CsgB. Proteins CsgE, CsgF, and CsgG are assembly factors required for the stabilization and transport of CsgA and CsgB to the cell surface. (b) Congo red-binding phenotype. Curli are amyloid fibers and bind the hallmark amyloid dyes Congo red and thioflavin T. Curliated *E. coli* grown on Congo red-containing agar medium take up the dye and stain red. Noncurliated cells do not. (c) High-resolution deep-etch electron micrographs of curliated *E. coli*. From Chapman MR, Robinson LS, Pinkner JS, *et al*. (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295: 851–855. Reprinted with permission from AAAS.

protein (CsgB) are secreted to the cell surface in a CsgG-dependent fashion. CsgE and CsgF are assembly factors required for the stabilization and transport of CsgA and CsgB. Transcriptional regulation of the curli operons is complex and responds to many environmental cues including temperature, pH, and osmolarity. The adhesive functionality is attributed to the main fiber subunit, CsgA.

Curli are also implicated in the binding of *E. coli* strains to plant surfaces and are expressed by many strains associated with food-borne illness, including the prototype strain *E. coli* O157:H7, which has caused several food-borne outbreaks in the United States and around the world. Although the exact nature of binding is still under investigation, curli production is sufficient to permit laboratory strains of *E. coli* to bind plant tissues, such as alfalfa. However, among pathogenic strains such as *E. coli* O157:H7, there appear to be redundant adhesion systems, and under the conditions tested, curli are not required for adhesion. Indeed, external conditions in the environment and in the host may differ as a function of time, and bacteria may depend more on one adhesive system than another in certain circumstances.

The curli bacterial adhesive fiber machinery has gained considerable attention since the discovery of curli as amyloid fibers in 2002. The sticky nature of curli amyloid fibers is like that of amyloid aggregates and plaques associated with eukaryotic amyloid disorders such as Alzheimer's and Parkinson's diseases. Thus, ongoing curli research that aims to elucidate structural features of curli assembly and the functional implications of curli-mediated adhesion may also provide valuable information to the exciting field of amyloid fiber biogenesis and aggregation.

Consequences of Microbial Adhesion in Human Disease

The critical first step in most infectious diseases requires physical contact between a bacterium and host cell. Bacterial adhesins mediate this binding event through the sophisticated adhesion mechanisms described above and allow the pathogen to gain a foothold in the host, initiating complex signaling cascades in both the pathogen and the host. Binding events can lead to extracellular colonization and invasion into underlying host cells. Adhesion is the first step that promotes the cascading sequelae of infectious diseases, particularly important in the pathogenesis of chronic infections including urinary tract infection (UTI), chronic otitis media (middle ear infection), and chronic lung infections.

E. coli and UTI

UPEC engage in an incredibly coordinated and regulated genetic and molecular cascade to assemble type 1 pili, as described above. UTIs are among the most common bacterial infections and nearly 50% of women will be afflicted by at least one UTI in their lifetime, with many experiencing recurrent UTIs. Virtually all clinical UPEC isolates express type 1 pili, enabling them to bind the mannose-containing host receptors, which results in invasion of host bladder epithelial cells. Inside urothelial cells, bacteria form large, densely packed, biofilm-like intracellular bacterial communities (IBCs) of morphologically coccoid bacteria, comprising up to 10^5 bacteria per superficial facet cell. In this intracellular niche, the pathogens are protected from antibodies, the flow of urine, and other host defenses. Yet, this is only the beginning of a sometimes life-long cycle of interactions between pathogen and host. IBC formation is not an end point or dead end for *E. coli*. Upon entry into superficial facet cells, UPEC activate a complex developmental cascade; UPEC eventually detach and disperse, or flux, from the IBC to initiate another round of IBC formation in other urothelial cells (**Figure 3**). Some fluxing bacteria form filaments, which are resistant to neutrophil phagocytosis. Filamentation facilitates survival of the bacteria and allows them to invade other epithelial cells. Even after acute infection is resolved and the urothelium is

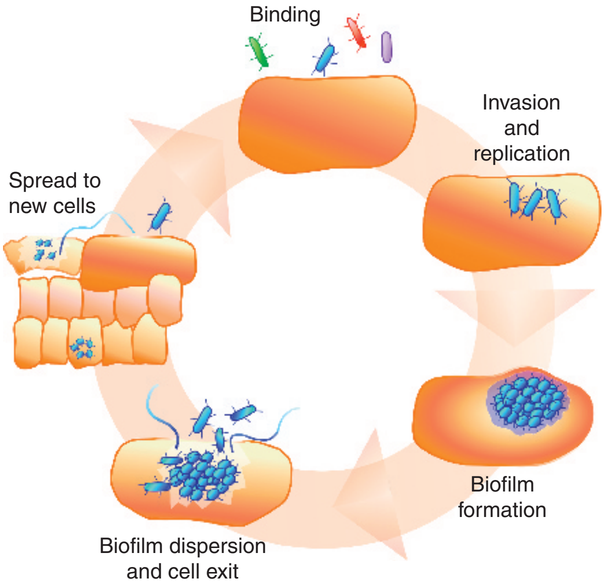

Figure 3 Pathogenic cascade of uropathogenic *E. coli* (UPEC). UPEC coordinate highly organized temporal and spatial events to colonize the urinary tract. UPEC bind to and invade the superficial umbrella cells that line the bladder lumen, where they rapidly replicate to form a biofilm-like intracellular bacterial community (IBC). In the IBC, bacteria find a safe haven, are resistant to antibiotics, and subvert clearance by innate host responses. UPEC can persist for months in a quiescent bladder reservoir following acute infection, and challenge current antimicrobial therapies. Quiescent bacteria can reemerge as pathogens from their protected intracellular niche and can be a source of recurrent urinary tract infections (UTIs).

seemingly intact, bacteria can remain within the bladder for many days to weeks regardless of standard antibiotic treatments. Thus, the ability of UPEC to adhere to and invade bladder cells appears to facilitate long-term bacterial persistence within the urinary tract.

P. aeruginosa and CF

P. aeruginosa has emerged as an opportunistic pathogen in several clinical settings, causing nosocomial infections such as pneumonia, UTIs, and bacteremia. *P. aeruginosa* adheres to the respiratory epithelium, leading to chronic lung infections in cystic fibrosis (CF) patients, responsible for the eventual pulmonary failure of most CF patients, typically by 37 years of age. Pilus-mediated adherence is important in the adhesion and early stages of epithelial colonization, and additional virulence factors contribute to the subsequent persistence in the lung. Alginate, for example, is a mucoid exopolysaccharide produced by *P. aeruginosa* that forms a matrix of 'slime' to surround a forming biofilm and anchors the cells to each other and to their host. Surrounded by alginate, the bacteria are protected from the host defenses and are often resistant to treatment with antibiotics.

Targeting Adhesion to Inhibit Bacterial Virulence

The ability to impair bacterial adhesion represents an ideal strategy to combat bacterial pathogenesis because of its importance early in the infectious process. In addition, adhesion is essential to the long-term persistence of bacteria in the pathogenic cascade of several infectious diseases. Moreover, the adhesion process can be targeted without placing life or death pressure on the bacterium, *per se*. Targeting bacterial virulence in this way is an alternative approach to the development of new therapeutics to disarm pathogens in the host that may offer reduced selection pressure for drug-resistant mutations. In addition, virulence-specific therapeutics could avoid the undesirable dramatic alterations of the host microbiota. Indeed, standard antibiotic treatment regimens may lead to the loss of symbiotic benefits and the proliferation of disease-causing opportunistic pathogens.

As emphasized earlier, pathogens are capable of presenting multiple adhesins that can be expressed differentially to permit binding in specific sites and at specific times over the course of a complex infectious cycle. Thus, it may be difficult to develop a universal class of antiadherence drugs. Nevertheless, several specific pathogenic adhesive strategies have emerged as hallmark requirements for virulence in certain infectious diseases, and represent amenable targets for drug discovery and development. Adhesion is sometimes just the first step of many in pathogenic cycles, yet targeting adhesion holds value even after an infection has been established. In biofilm-associated infections, for example, drug development strategies include attempts to induce the dispersal of bacteria from the biofilm and to inhibit the chemical signaling necessary to encourage new biofilm formation. In UTI, the fluxing bacteria are capable of readhering to new host cells, gaining a foothold and potentially invading a new cell to remain undetected until drug pressure subsides and conditions encourage replication and new intracellular biofilm formation. Thus, strategies to prevent microbial adhesion are being considered in combination therapies to both prevent and treat infectious diseases.

Carbohydrate derivatives of host ligands have demonstrated efficacy in blocking the adhesive properties of *E. coli* expressing type 1 and also P pili in biophysical and hemagglutination assays. This approach of using soluble carbohydrates or mimics recognized by the bacterial lectin can be readily extended to other adherent organisms by tailoring the antiadhesive compounds to their receptor specificities.

'Pilicides' are a class of pilus inhibitors that target chaperone function. A new class of pilicides, based on a bicyclic 2-pyridone scaffold, inhibit the assembly of both type 1 and P pili in *E. coli* (**Figure 4**). The potent molecules inhibit an essential protein–protein interaction between chaperone and usher, required for pilus biogenesis. Chaperone–usher systems are highly conserved among various bacteria including *Salmonella*, *Haemophilus*, *Klebsiella*, and *Yersinia* and it is possible, although not yet demonstrated, that pilicides may exert broad-spectrum activity and be effective against several Gram-negative pathogens.

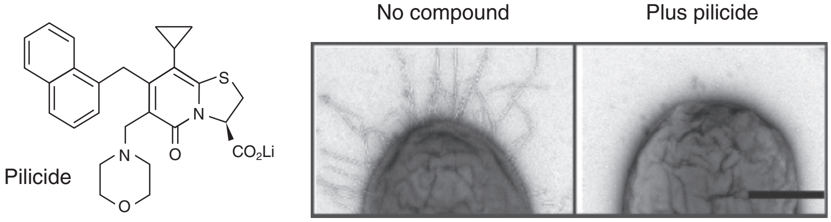

Figure 4 Targeting microbial adhesion. Rationally designed 'pilicides' inhibit pilus biogenesis by disrupting chaperone–usher protein interactions and reduce piliation levels dramatically. Electron micrographs reproduced from Pinkner JS, Remaut H, Buelens F, *et al*. (2006) Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America* 103: 17897–17902. Copyright (2006) National Academy of Sciences, U.S.A.

Compounds have been identified that target the two-component signaling system, AlgR2/AlgR1, that controls the synthesis of alginate by *P. aeruginosa*. Alginate is a key component of the protective exopolysaccharide coat, critical to *P. aeruginosa* adherence, biofilm formation, and CF pathogenesis. The inhibitors of alginate synthesis could be therapeutically employed to render the pathogen more susceptible to host defenses or to standard antibiotics currently in use, and thus could be effective also in combination therapy. The ability to inhibit microbial adhesion and thus prevent subsequent pathogenic processes holds enormous therapeutic potential and promises to improve the treatment of numerous infectious diseases.

Acknowledgments

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Agrobacterium and Plant Cell Transformation

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Defining Statement
Introduction
Overview of Infection Process
Ti Plasmid
Chromosomally Encoded Virulence Genes
T-DNA Processing
VirB/D4 System, a Member of the Type IV Secretion Family
Substrate Transfer Through the Plant Cell
Agrobacterium Host Range and Genetic Engineering
Conclusions
Further Reading

Glossary

autoinducer An acylhomoserine lactone secreted from bacteria, which, under conditions of high cell density, passively diffuses across the bacterial envelope and activates transcription.

border sequences 25-bp direct, imperfect repeats that delineate the boundaries of T-DNA.

conjugation Transfer of DNA between bacteria by a process requiring cell-to-cell contact.

conjugative pilus An extracellular filament encoded by a conjugative plasmid involved in establishing contact between plasmid-carrying donor cells and recipient cells.

mobilizable plasmid Conjugal plasmid that carries an origin of transfer (*oriT*) but lacks genes coding for its own transfer across the bacterial envelope.

T-DNA Segment of the *Agrobacterium* genome transferred to plant cells.

transconjugant A cell that has received a plasmid from another cell as a result of conjugation.

transfer intermediate A nucleoprotein particle composed of a single-strand of the DNA destined for export and one or more proteins that facilitate DNA delivery to recipient cells.

type IV secretion system A conserved family of macromolecular translocation systems evolutionarily related to conjugation systems for translocating DNA or protein effector molecules between prokaryotic cells or to eukaryotic hosts.

Abbreviations

AAI	autoinducer
ABC	ATP-binding cassette
AHL	acylhomoserine lactone
CP	coupling protein
Dtr	DNA transfer and replication
GFP	green fluorescent protein
GGI	gonococcal genetic island
IM	inner-membrane
Mpf	mating pair formation
NLS	nuclear localization sequences
OD	*overdrive*
OM	outer membrane
SR	substrate receptor
T-DNA	transferred DNA
Ti	tumor-inducing
TMS	transmembrane segments
TrIP	transfer DNA immunoprecipitation
T4S	type IV secretion
VBTs	VirB2-interacting proteins

Defining Statement

Agrobacterium tumefaciens transfers oncogenic DNA (T-DNA) to susceptible plant cells, causing formation of tumors called Crown galls. This is a multistage infection process involving sensory recognition of specific plant signals, attachment to the plant host, induction of a virulence regulon, and T-DNA processing, transfer, and integration into the plant genome.

Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium with the ability to infect plants through a process that involves delivery of a specific segment of its genome to the nuclei of susceptible plant cells. The transferred DNA (T-DNA) is a discrete region of the bacterial genome delimited by 23 base pair (bp) direct repeats carried by the tumor-inducing (Ti) plasmid. The T-DNA is important for infection because it codes for genes that, when expressed in the plant cell, disrupt plant cell growth and division events.

However, this oncogenic DNA can be excised from the transferred DNA and replaced by virtually any gene of interest for *A. tumefaciens*-mediated engineering of a wide array of plant species. The discovery that *A. tumefaciens* is a natural and efficient DNA delivery vector spawned an entire new industry of plant genetic engineering, which today has many diverse goals ranging from crop improvement to the use of plants as 'pharmaceutical factories' for high-level production of biomedically important proteins. Because of the dual importance of *Agrobacterium* as a plant pathogen and as a DNA delivery system, an extensive literature has emerged describing numerous aspects of the infection process and the myriad of ways this organism has been exploited for plant genetic engineering. Here, I will summarize recent findings pertaining to the mechanistic details of *vir* gene induction, T-DNA processing and transfer, and T-DNA movement and integration in the plant host.

Overview of Infection Process

Agrobacterium species are commonly found in a variety of environments including cultivated and nonagricultural soils, plant roots, and even plant vascular systems. Despite the ubiquity of *Agrobacterium* species in soil and plant environments, only a small percentage of isolates are pathogenic. Two species are known to infect plants by delivering DNA to susceptible plant cells. *A. tumefaciens* is the causative agent of crown gall disease, a neoplastic disease characterized by uncontrolled cell proliferation and formation of unorganized tumors. *Agrobacterium rhizogenes* induces formation of hypertrophies with a hairy-root appearance referred to as 'hairy-root' disease. The pathogenic strains of both the species possess large plasmids Ti and Ri, respectively, that encode most of the genetic information required for DNA transfer to susceptible plant cells. The basic infection process is similar for both the species, although the gene composition of the transferred DNA differs, and, therefore, the outcome of the infection. *Agrobacterium* has been widely viewed as the only bacterial genus capable of transferring genes to plants, but in fact other members of the alphaproteobacteria can transform plants when carrying an *Agrobacterium* Ti plasmid. The plant symbionts *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti*, were found to transfer T-DNA, albeit inefficiently, into the chromosomes of tobacco, *Arabidopsis*, and rice plants. This discovery highlights the importance of the Ti-plasmid-encoded virulence (*vir*) genes and certain conserved chromosomal loci among these alphaproteobacteria for infection. Here, I will focus on *Agrobacterium*-mediated transformation as a model for understanding the requirements for interkingdom DNA transfer.

Agrobacterium-mediated transformation can be depicted as a multistage process involving (1) sensory perception of plant signals and induction of virulence genes, (2) establishment of physical contact between *A. tumefaciens* and the plant host, (3) processing of T-DNA and protein effectors for translocation, (4) translocation across the bacterial envelope via a dedicated secretion channel, (5) movement of substrates through the plant cell cytoplasm to the nucleus, (6) integration of T-DNA into the plant genome, and (7) expression of T-DNA genes (see **Figure 1**). With the exception of attachment, early stages of infection are mediated by genes encoded by the Ti plasmid.

Ti Plasmid

Ti plasmids range in size from ~180 to as many as 800 kilobases (kb). Two regions of the Ti plasmid contribute to infection (**Figure 2**). The first is the T-DNA, typically a segment of 20–35 kb delimited by 25-bp directly repeated border sequences. The T-DNA harbors genes that are expressed exclusively in the plant cell. Transcription of T-DNA in the plant cell produces 3′ polyadenylated RNA typical of eukaryotic RNA message that is translated in the cytoplasm. The second region of the Ti plasmid involved in infection harbors the genes responsible for sensory recognition of plant signals, T-DNA processing for transfer, and substrate transfer across the bacterial envelope. Two additional regions of the Ti plasmid code for functions that are not essential for the T-DNA transfer process *per se*, but are nevertheless intimately associated with the overall infection process. One of these regions harbors genes involved in catabolism of novel amino acid derivatives termed opines that *A. tumefaciens* induces plants to synthesize as a result of T-DNA transfer. The other encodes Ti plasmid transfer functions for distributing copies of the Ti plasmid and its associated virulence factors to other *A. tumefaciens* cells by a process termed as conjugation. Intriguingly, a novel regulatory cascade involving chemical signals released both from the transformed plant cells and the infecting bacterium serves to activate conjugative transfer of the Ti plasmid among *A. tumefaciens* cells residing in the vicinity of the plant tumor.

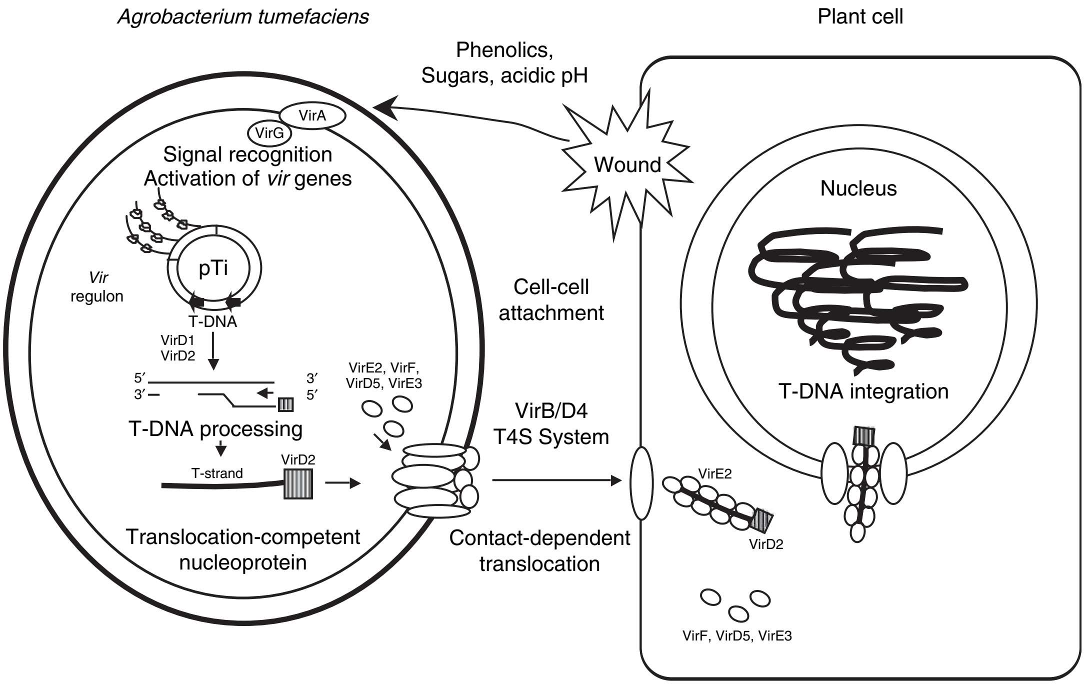
Agrobacterium tumefaciens
Plant cell
Phenolics,
Sugars, acidic pH
VirA
VirG
Signal recognition
Activation of vir genes
Wound
Nucleus
Vir
regulon
pTi
T-DNA
VirD1
VirD2
Cell-cell
attachment
5′
3′
3′
5′
VirE2, VirF,
VirD5, VirE3
T-DNA processing
VirB/D4
T4S System
T-DNA integration
T-strand
VirD2
VirE2
VirD2
Translocation-competent
nucleoprotein
Contact-dependent
translocation
VirF, VirD5, VirE3

Figure 1 Overview of *Agrobacterium tumefaciens* infection process. Upon activation of the VirA/VirG two-component signal transduction system by signals released from wounded plant cells, a single-strand transferred DNA (T-DNA) is processed from the Ti plasmid and delivered as a nucleoprotein complex (T-complex) to plant nuclei. Expression of T-DNA genes in the plant results in loss of cell growth control and tumor formation (see text for details).

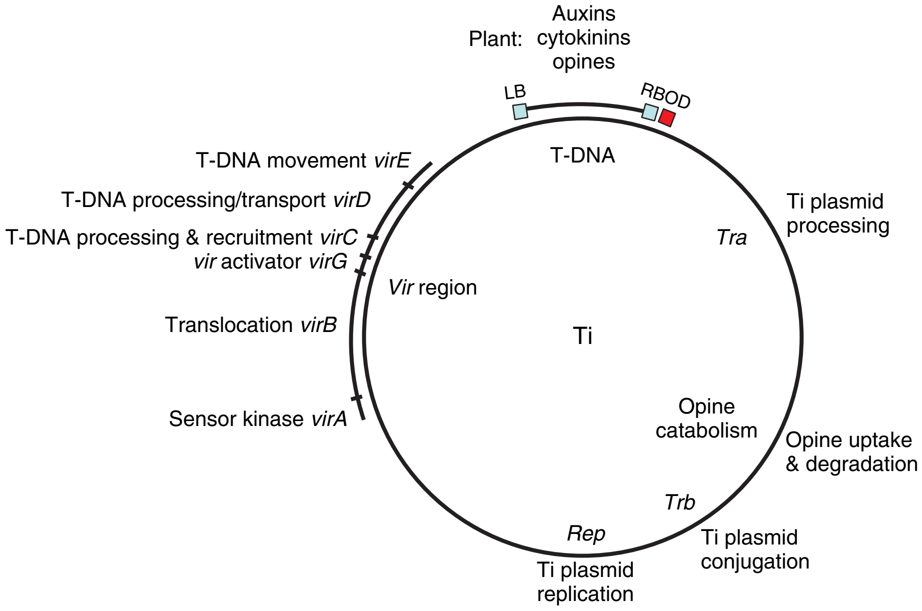
Plant:
Auxins
cytokinins
opines
LB
RBOD
T-DNA
T-DNA movement virE
T-DNA processing/transport virD
T-DNA processing & recruitment virC
vir activator virG
Translocation virB
Sensor kinase virA
Vir region
Ti
Ti plasmid
processing
Tra
Opine
catabolism
Opine uptake
& degradation
Trb
Rep
Ti plasmid
conjugation
Ti plasmid
replication

Figure 2 Regions of the Ti plasmid that contribute to infection (*vir* region and T-DNA), cell survival in the tumor environment (opine catabolism), and conjugal transfer of the Ti plasmid to recipient agrobacteria (*tra* and *trb*). The various contributions of the *vir* gene products to T-DNA transfer are listed. T-DNA, delimited by 25-bp border sequences (blue boxes; RB, right border; LB, left border) codes for biosynthesis of auxins, cytokinins, and opines in the plant. OD, *overdrive* sequence (red box) that enhances VirD2-dependent processing at the T-DNA border sequences.

T-DNA

The T-DNA is delimited by DNA repeats termed as border sequences (**Figure 2**). Flanking one border is a sequence termed as *overdrive* that functions to stimulate the T-DNA-processing reaction. All DNA between the border sequences can be excised and replaced with genes of interest, and *A. tumefaciens* will still efficiently transfer the engineered T-DNA to plant cells. This shows that the border sequences are the only *cis* elements required for T-DNA transfer to plant cells. Additionally, genes encoded on the T-DNA play no role in the movement of T-DNA to plant cells. The T-DNA genes instead code for synthesis of enzymes within transformed plant cells. Oncogenes synthesize enzymes involved in the synthesis of two plant growth regulators, auxins and cytokinins. Production of these plant hormones results in a stimulation in cell division and a loss of cell growth control leading to the formation of characteristic crown gall tumors. Other enzymes catalyze the synthesis of novel amino acid derivatives termed as opines. The pTiA6 plasmid, for example, carries two T-DNA's that code for genes involved in synthesis of octopines – a reductive condensation product of pyruvate and arginine. Other Ti plasmids carry T-DNAs that code for nopalines, derived from α-ketoglutarate and arginine, and still others code for different classes of opines.

Plants cannot metabolize opines. However, the Ti plasmid carries opine catabolism genes that are responsible for the active transport of opines and their degradation, thus providing a source of carbon and nitrogen for the bacterium. The 'opine concept' was developed to rationalize the finding that *A. tumefaciens* evolved as a pathogen by acquiring the ability to transfer DNA to plant cells. According to this concept, *A. tumefaciens* adapted a DNA conjugation system for interkingdom DNA transport to incite opine synthesis in its plant host. The cotransfer of oncogenes ensures that transformed plant cells proliferate, resulting in enhanced opine synthesis. The tumor, therefore, is a rich chemical environment favorable for growth and propagation of the infecting *A. tumefaciens*. Of further interest, a given *A. tumefaciens* strain generally catabolizes only those opines that it incites plant cells to synthesize. This ensures a selective advantage of the infecting bacterium over other *A. tumefaciens* strains that are present in the vicinity of the tumor.

Opine Catabolism

The regions of Ti plasmids involved in opine catabolism code for three functions related to opine catabolism. The first is a regulatory function controlling expression of opine transport and catabolism genes. For the octopine catabolism region of plasmid pTiA6, the regulatory protein is OccR, a member of the family of LysR transcription factors. OccR positively regulates expression of the *occ* genes involved in octopine uptake and catabolism by inducing a bend in the DNA at the OccR-binding site. Octopine modulates OccR regulatory activity by altering both the affinity of OccR for its target site and the angle of the DNA bend. The regulatory protein for the nopaline catabolism region of plasmid pTiC58 is AccR. In contrast to OccR, AccR functions as a negative regulator of *acc* genes involved in nopaline catabolism.

Several other genes transcribed from a single promoter specify functions for opine transport and catabolism. At the proximal end of the operon are transport genes mediating opine-specific binding and uptake. Typically, one or more of these genes encode proteins homologous to energy-coupling proteins found associated with the so-called ATP-binding cassette (ABC) superfamily of transporters. The ABC transporters are ubiquitous among bacterial and eukaryotic cells, and provide a wide variety of transport functions utilizing the energy of ATP hydrolysis to drive the transport reaction. At the distal end of the operon are genes whose products cleave opines to their parent compounds for use as carbon and nitrogen sources for the bacterium.

Ti Plasmid Conjugation

The Ti plasmid transfer (*tra* and *trb*) functions direct the conjugative transfer of the Ti plasmid to bacterial recipient cells (**Figure 2**). The transfer genes of conjugative plasmids code for DNA-processing factors and a translocation system. The Ti plasmid transfer system is related in sequence and function to other plasmid transfer systems, as well as dedicated protein translocation systems. These systems are now classified as type IV secretion (T4S) systems (see below).

A regulatory cascade activates Ti plasmid transfer under conditions of high cell density (**Figure 3**). This regulatory cascade initiates when *A. tumefaciens* imports opines released from plant cells. For the octopine pTiA6 plasmid, OccR acts in conjunction with octopine to activate transcription of the *occ* operon. Although the majority of the *occ* operon codes for octopine transport and catabolism functions, the distal end of the *occR* operon encodes a gene for a transcriptional activator termed TraR. TraR is related to LuxR, an activator shown nearly 20 years ago to regulate synthesis of an acylhomoserine lactone (AML) termed as *autoinducer*. Cells that synthesize autoinducer molecules secrete these molecules into the environment. At low cell densities, autoinducer is in low concentration, whereas at high cell densities this substance accumulates in the surrounding environment and passively diffuses back into the bacterial cell to activate transcription of a defined set of genes. In the case of *A. tumefaciens*, the autoinducer is an *N*-3-(oxo-octonoyl)-L-homoserine lactone termed *Agrobacterium* autoinducer (AAI). AAI acts in conjunction with TraR to activate transcription of the Ti plasmid *tra* genes as well as *traI* whose product mediates synthesis of AAI. Therefore, synthesis of TraR under conditions of high cell density creates a positive-feedback loop whereby a TraR–AAI complex induces transcription of TraI, which, in turn, results in enhanced synthesis of more AAI. This regulatory cascade, involving

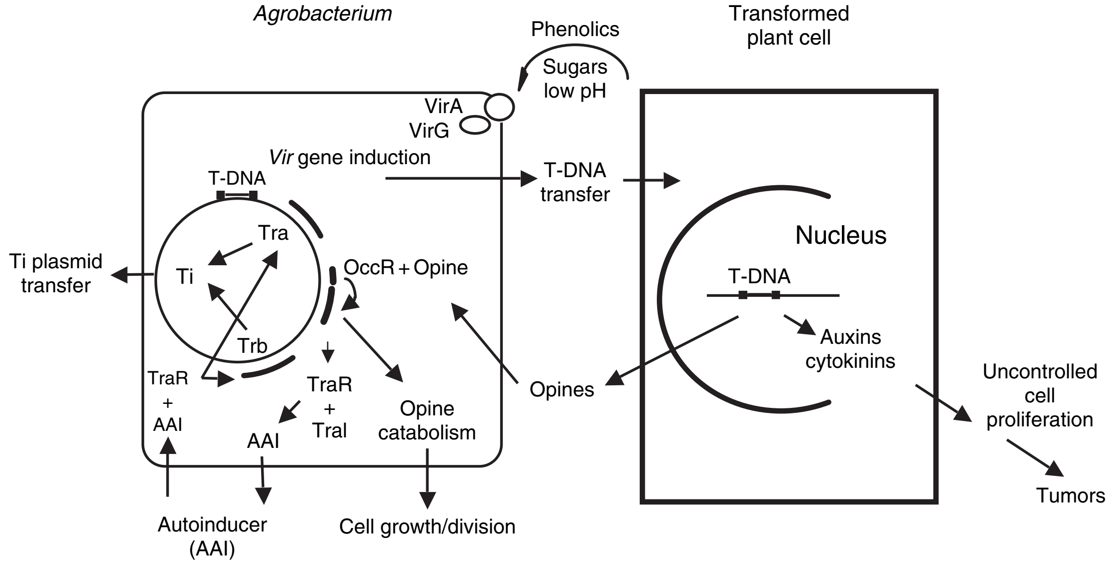

Figure 3 A schematic of chemical signaling events between *Agrobacterium* and the transformed plant cell. Signals released from wounded plant cells initiate the infection process leading to tumor formation. Opines released from wounded plant cells activate opine catabolism functions for growth of infecting bacteria. Opines also activate synthesis of TraR for autoinducer (AAI) synthesis. TraR and AAI at a critical concentration activate the Ti plasmid conjugation functions (see text for details).

opine-mediated expression of *traR* and TraR-AAI mediated expression of Ti plasmid transfer genes under conditions of high cell density, has the net effect of enhancing Ti plasmid transfer in the environment of the plant tumor. This complex regulatory system likely evolved to maximize the number of Ti-plasmid-carrying bacterial cells in the vicinity of the plant wound site.

AAI-mediated activation of Ti plasmid transfer is also negatively controlled. For example, TraR activity is antagonized by two proteins, TraM and TrlR. TraM interacts with the C-terminus of TraR, which inactivates TraR and disrupts TraR–DNA complexes. TrlR is a truncated form of TraR that suppresses TraR activity through formation of inactive heterodimers. In addition, an AAI signal turnover system is composed of *attJ*, a regulatory gene, and *attM* whose product is an *N*-acylhomoserine lactone-lactonase that hydrolyzes the lactone ring of AAI. Lactonase production suppresses AAI-dependent expression of conjugation genes as a means of fine-tuning plasmid transfer in response to changes in cell growth and density.

vir Genes

The Ti plasmid carries a ∼35-kb region harboring a number of operons involved in T-DNA transfer (**Figure 1**). Some operons have a single open reading frame, while others code for up to 11 open reading frames. The products of the *vir* region direct events within the bacterium that must precede export of a copy of the T-DNA to plant cells: (1) a VirA/VirG two-component regulatory system induces expression of the *vir* genes in response to perception of plant-derived signals, (2) VirC and VirD proteins process T-DNA into a nucleoprotein particle for delivery to plant nuclei, and (3) a T4S system composed of VirB proteins and VirD4 translocates the T-DNA transfer intermediate and effector proteins across the bacterial envelope (**Figure 2**).

Infection is initiated when bacteria sense and respond to an array of signals, including specific classes of plant phenolic compounds, aldose monosaccharides, low PO_4, and an acidic pH that are present at a plant wound site (**Figure 1**). The VirA/VirG signal transduction system together with ChvE, a periplasmic sugar-binding protein, mediates recognition of plant phenolics and sugars. VirA was one of the first described of what now is recognized as a very large family of sensor kinases identified in bacteria and more recently in eukaryotic cells. The members of this protein family are typically integral membrane proteins with an N-terminal extracytoplasmic domain. Upon sensory perception, the kinase autophosphorylates at a conserved histidine residue, then transferring the phosphate group to a conserved aspartate residue on the second component of this transduction pathway, the response regulator. The phosphorylated response regulator coordinately activates transcription of several operons whose products mediate a specific response to the inducing environmental signal. For the *A. tumefaciens vir* system, the response regulator is VirG, and phosphorylated VirG activates transcription of six essential *vir* operons as well as a number of other Ti plasmid and chromosomally encoded operons whose products are probably important for infection of certain plant species or under certain environmental conditions. The VirA/VirG two-component system also activates expression of the *repABC* genes responsible for replication of the Ti plasmid. Plant signals thus enhance Ti plasmid copy number and, consequently, virulence potential upon perception of environmental conditions favorable for interkingdom DNA transfer.

VirA senses most of the plant-derived signals listed above. The most important signal molecules are phenols that carry an *o*-methoxy group. The type of substitution at the *para* position distinguishes strong inducers such as acetosyringone from weaker inducers such as ferulic acid and acetovanillone. A variety of aldose monosaccharides, including glucose, galactose, arabinose, and the acidic sugars D-galacturonic acid and D-glucuronic acid, strongly enhance *vir* gene induction. The inducing phenolic compounds as well as the monosaccharides are secreted intermediates of biosynthetic pathways involved in cell wall repair. As such, the presence of these compounds is a general feature of most plant wounds and likely contributes to the extremely broad host range of *A. tumefaciens*. VirA functions as a homodimer, and a model that VirA interacts directly with inducing molecules that diffuse across the outer membrane (OM) into the periplasm is supported by genetic experiments though direct evidence for signal binding is lacking. Sugar-mediated inducing activity occurs via an interaction between sugars and the periplasmic sugar-binding protein ChvE. In turn, ChvE sugar interacts with the periplasmic domain of VirA to induce a conformational change that increases the sensitivity of VirA to phenolic inducer molecules. A periplasmic domain of VirA is also implicated in recognition of acidic pH, though the physical mechanism of pH perception is unknown.

On the basis of recent crystallographic analysis of CheY, a homologue of VirG, phosphorylation of this family of response regulators is thought to induce a conformational change. Phospho-VirG activates transcription of the *vir* genes by interacting with a *cis*- acting regulatory sequence (TNCAATTGAAAPy) called the *vir* box located upstream of each of the *vir* promoters. Interestingly, both nonphosphorylated and phosphorylated VirG bind to the *vir* box, indicating that a phosphorylation-dependent conformation is necessary for a productive interaction with components of the transcription machinery.

Chromosomally Encoded Virulence Genes

Most studies of the *A. tumefaciens* infection process have focused on the roles of Ti plasmid genes in T-DNA transfer, but several essential and ancillary chromosomal genes also contribute to *A. tumefaciens* virulence. Although mutations in these genes are often pleiotropic, they generally function to regulate *vir* gene expression or mediate attachment to plant cells. This latter activity will be described in the section titled 'Attachment to plant cells'.

Regulators of *vir* Gene Expression

At least three groups of chromosomal genes activate or repress *vir* gene expression. As described above, the periplasmic sugar-binding protein ChvE complexed with any of a wide variety of monosaccharides induces conformational changes in VirA allowing it to interact with phenolic inducers. *chvE* mutants are severely compromised for T-DNA transfer, but they also show defects in chemotaxis toward sugars. ChvE thus appears to play a dual role in the infection process, by promoting bacterial chemotaxis toward nutrients and by enhancing the efficiency of opine-encoding T-DNA to plant cells.

A second locus codes for Ros, a novel prokaryotic zinc finger protein that transcriptionally represses certain *vir* operons. As described below, the VirC and VirD operons contribute to the T-DNA processing reaction. Although the promoters for these operons are subject to positive regulation by the VirA/VirG transduction system, they are also negatively regulated by the Ros repressor. Ros binds to a 9-bp inverted repeat, the *ros* box residing upstream of these promoters. In the presence of plant signals, Ros repression is counteracted by the VirA/VirG induction system, but in the absence of plant signals, Ros binding to the *virC* and *virD* promoters prevents the T-DNA-processing reaction. In addition to repressing expression of T-DNA processing genes in the absence of a suitable plant host, Ros prevents premature expression of the T-DNA oncogenes in the bacterium.

Finally, a two-component regulatory system, distinct from the VirA/VirG system, senses environmental signals and mounts a behavioral response by modulating gene expression. ChvG is the sensor kinase and ChvI is the response regulator. Null mutations in genes for these proteins block *vir* gene induction or growth of cells at an acidic pH of 5.5. The molecular basis underlying the effect of the ChvG and ChvI proteins on *vir* gene expression is presently unknown.

T-DNA Processing

One of the early events following attachment to plant cells and activation of *vir* gene expression in response to plant signals involves the processing of T-DNA into a form that is competent for transfer across the bacterial cell envelope and translocation through the plant plasma membrane, cytosol, and nuclear membrane. The prevailing view strongly supported by molecular and genetic data is that T-DNA is transferred as a nucleoprotein particle composed of a single-stranded DNA molecule (T-strand) covalently attached to a nicking enzyme (see below).

Roles of VirD2 Relaxase in T-DNA Processing and Transfer

It is now widely accepted that DNA-processing reactions associated with T-DNA transfer are equivalent to those mediating bacterial conjugation. In the generalized reaction, a set of proteins termed as the DNA transfer and replication (Dtr) proteins assemble at an origin-of-transfer (*oriT*) sequence to generate a nucleoprotein complex termed as the *relaxosome*. One component of the relaxosome, the relaxase, cleaves and remains covalently associated with the 5′ end of the DNA strand destined for transfer (T-strand). The T-strand is unwound from its template by a strand displacement reaction, generating the translocation-competent relaxase-T-strand substrate. In *A. tumefaciens*, the VirD2 relaxase generates nicks at *oriT*-like sequences located in the T-DNA border repeats. VirD2 remains covalently bound to the 5′ phosphoryl end of the nicked T-DNA via conserved tyrosine residue Tyr-29. Purified VirD2 catalyzes cleavage of oligonucleotides bearing a T-DNA nick site. However, an ancillary protein, VirD1, is essential for nicking *in vitro* when the nick site is present on a supercoiled, double-stranded plasmid.

In addition to *oriT* nicking, the relaxase component of the conjugative transfer intermediate is thought to participate in translocation of substrate DNA by supplying a signal motif recognizable by the transport machinery. VirD2 and other relaxases carry a motif at their extreme C termini that is devoid of secondary structure and rich in positively charged amino acids, particularly arginines. This motif is also present at the C-termini of protein substrates of the VirB/D4 T4S system and, as expected, mutations in the signal motif of one such substrate, VirF, block translocation. The charged motif likely confers recognition of the substrate by the secretion channel, as suggested by evidence that the VirD2-T-strand complex, as well as another protein substrate, VirE2, interact with the VirD4 substrate receptor (SR). Moreover, when the C-terminal fragment of VirE2 is fused to the green fluorescent protein (GFP), it mediates binding of the reporter protein to VirD4 in living cells. Early studies supplied evidence for 5′-3′ unidirectional transfer of the T-strand, which is also compatible with the notion that the relaxase serves to pilot the attached T-strand through the secretion channel.

Roles of Ancillary Processing Factors in T-DNA Processing and Transfer

Although VirD2 catalyzes nicking of T-DNA substrates *in vitro*, border cleavage *in vivo* requires accessory proteins including VirD1, VirC1, and VirC2 proteins. Early studies showed that VirC1 binds the *overdrive* sequence located next to the right border repeat sequences of octopine-type Ti plasmids. A recent quantitative analysis established that both VirC1 and VirC2 are required for synthesis of as many as 50 copies of the T-DNA transfer intermediate per cell within a 24-h induction period. A mutation in an invariant Lys residue in the Walker A nucleotide triphosphate binding motif of VirC1 (VirC1K15Q) abolished the stimulatory effect of VirC1 on T-strand production, suggesting that VirC1's activity is regulated by ATP binding or hydrolysis.

VirC1 is related to the ParA/MinD family of ATPases, which mediate partitioning of chromosomes and plasmids during cell division. Very interestingly, VirC1 localizes at cell poles, which is also the site of VirB/D4 machine assembly (see below). Besides stimulating the conjugative processing reaction, polar-localized VirC1 supplies another important function to stimulate substrate translocation. It recruits the VirD2-T-strand nucleoprotein particle to the VirB/D4 transfer machine. Such stimulatory functions associated with conjugation have not been described previously for other ParA/MinD homologues, but note that many mobile elements – both integrated and extrachromosomal – encode ParA/MinD homologues. In future studies, it will be interesting to determine whether these proteins provide VirC1-like functions to couple processed DNA substrates with their cognate transfer machines.

VirB/D4 System, a Member of the Type IV Secretion Family

A. tumefaciens translocates the T-complex as well as effector proteins through a dedicated secretion channel assembled from 11 VirB subunits and VirD4. The VirB proteins are termed as the mating pair formation (Mpf) proteins, and VirD4 the SR, also termed as the coupling protein (T4CP). As discussed above for the Dtr-processing factors, the VirB and VirD4 proteins are related in sequence and function to subunits of conjugation systems, further underscoring the notion that *A. tumefaciens* adapted an ancestral conjugation system to deliver effector macromolecules to plants during infection.

The VirB/D4 system and other conjugation machines of Gram-negative and -positive bacteria are members of a large family of translocation systems termed as T4S systems (**Figure 4**). In addition to the conjugation machines, the T4S family encompasses two other subfamilies. One,

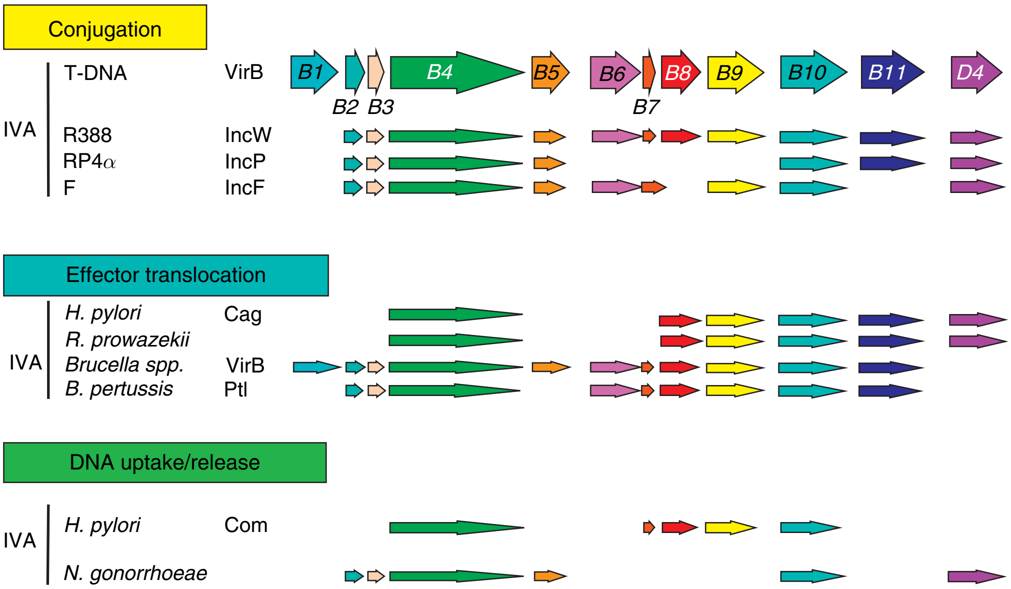

Figure 4 Alignment of genes encoding related components of the T4S systems. Of the 11 VirB proteins, those encoded by *virB2* through *virB11* and *virD4* are essential for T-complex transport to plant cells. Ancestrally related conjugation systems mediate interbacterial transfer of DNA. Effector translocation systems function to secrete proteins to eukaryotic cells during the course of infection by many medically important bacterial pathogens. A third subfamily of T4S systems, designated as the DNA uptake/release systems, take up DNA from the extracellular milieu or release DNA to the environment.

termed as the 'DNA uptake and release' systems, function independently of contact with a target cell to take up DNA from the extracellular milieu, as exemplified by the *Helicobacter pylori* ComB competence system, or to release DNA to the milieu, as exemplified by a chromosomally encoded F-like transfer system carried on the gonococcal genetic island (GGI) of *Neisseria gonorrhoeae.* As with the conjugation machines, these systems promote genetic exchange and, therefore, also represent potential mechanisms for transfer of survival traits during infection.

The third subfamily, the 'effector translocator' systems, play indispensable roles in the infection processes of many prominent pathogens of plants and mammals. These machines can be viewed as 'injectisomes', reminiscent of the needle complexes elaborated by type III secretion (T3S) machines, because they deliver their substrates through direct contact with the eukaryotic target cell. The list of pathogens dependent on effector translocators for disease progression includes at least two phytopathogens, *A. tumefaciens* and *Burkholderia cepacia*, plant symbionts such as *S. meliloti*, and several pathogens of mammals including *H. pylori, Legionella pneumophila*, and *Brucella* and *Bartonella* species. *Bordetella pertussis* uses an effector translocator as well, but this system functions as a true exporter to deliver its toxin substrate to the extracellular milieu. Related systems of several additional pathogens are also implicated in the trafficking of substrates to eukaryotic cells, and thus the list of T4S effector translocators continues to grow.

The T4S systems are classified on the basis of extensive sequence similarities with subunits of conjugation machines (**Figure 4**). Although these systems are functionally versatile in terms of the substrates and target cells to which substrates are delivered, they share a number of common structural and functional features that distinguish them from other known bacterial translocation systems.

VirB/D4 Type IV System: Machine Architecture

The VirB/D4 system is composed of two surface structures – a secretion channel and a conjugative pilus (**Figure 5**). At this time, there is no high-resolution structure for either structure. Nevertheless, fairly comprehensive architectural models of the T4S system can be generated through topological, structural, and interaction studies of machine subunits. These studies have supplied evidence for at least three stable subassemblies of VirB/D4 components.

Energy subcomplex – VirD4, VirB4, VirB11

VirD4, VirB11, and VirB4 are the three energetic components of the VirB/D4 T4SS. Each of these subunits possesses a characteristic nucleoside triphosphate binding site (Walker A) motif required for substrate translocation. Mutations in the Walker A motifs invariably abolish substrate translocation, strongly indicating that ATP binding drives machine assembly or function.

Structures of soluble domains of two VirD4-like proteins have now been solved by X-ray crystallography, one

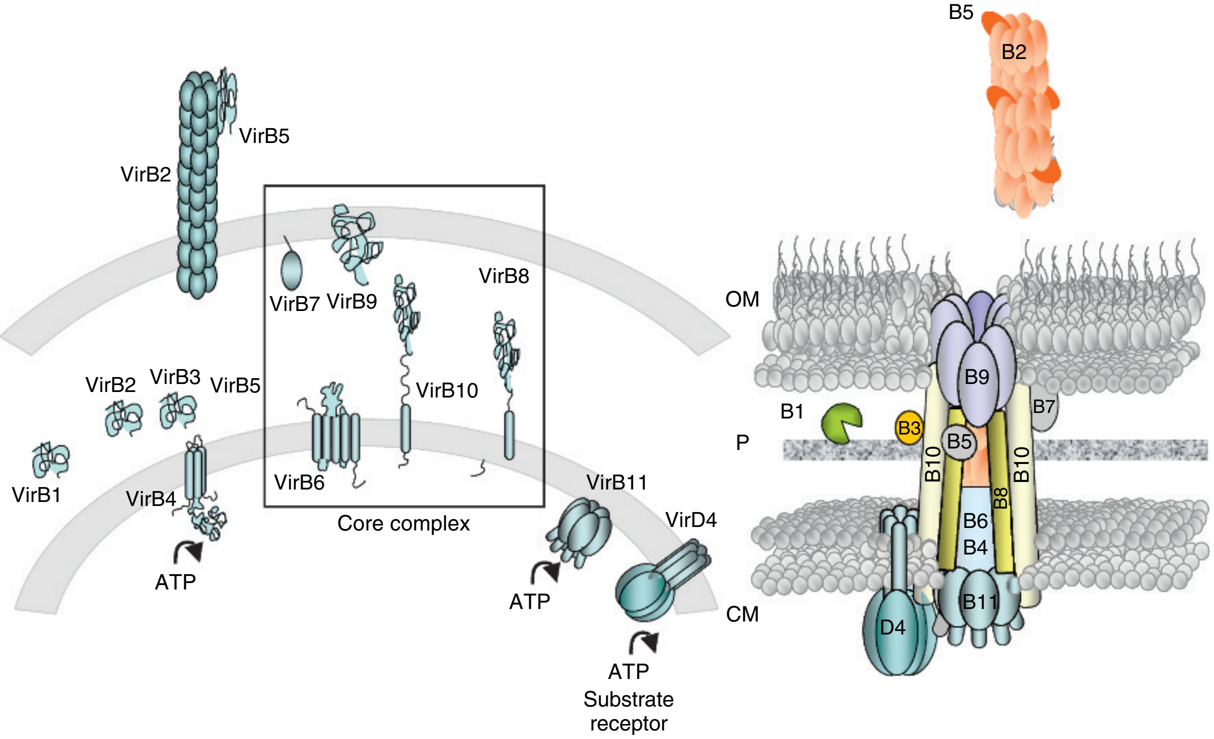

Figure 5 Topologies, structures, and cellular localizations of the VirB/D4 T4S subunits. The *Agrobacterium tumefaciens* VirB/D4 T4S system localizes at the cell poles and is postulated to assemble as a transenvelope complex through which substrates pass to the cell surface. The three ATPases energize machine assembly and substrate transfer and a stable 'core' complex nucleates machine assembly. All of the VirB proteins are required to build the T pilus; the VirB proteins plus VirD4 are required for substrate secretion. The T pilus is sloughed from the cell surface and is not essential for DNA or protein translocation.

of TrwC encoded by plasmid R388 and one of *Escherichi coli* FtsK. TrwC presents as six equivalent protomers assembled as a spherical particle of overall dimensions 110 Å in diameter and 90 Å in height. The overall structure bears a striking resemblance to the F1-ATPase $\alpha3\beta3$ heterohexamer, whereas the structure of the soluble domain closely resembles DNA ring helicases and other proteins such as FtsK that translocate along ss- or dsDNA. The FtsK structure is slightly larger with an outer diameter of 120 Å and a central annulus of 30 Å. The predicted structure is a dodecamer composed of two hexamers stacked in a head-to-head arrangement. As shown by electron microscopy imaging, dsDNA runs through the FtsK annulus, providing a structural view of a previously described ATP-dependent translocase activity. VirD4, therefore, functions as a receptor for the T-DNA and protein substrates of the VirB/D4 T4S system, and it might also function as an inner-membrane (IM) translocase, though this needs to be explored.

VirB11 is a member of a large family of ATPases associated with systems dedicated to secretion of macromolecules. Purified homologues TrbB, TrwC, *H. pylori* HP0525, and *Brucella suis* VirB11 assemble as homohexameric rings discernible by electron microscopy, and the last two also by X-ray crystallography. These structures present as double-stacked rings formed by the N- and C-terminal halves and a central cavity of ~50 Å in diameter. VirB11 associates peripherally but tightly with the IM of *A. tumefaciens*, and there is some evidence for ATP regulation of membrane binding. The role of VirB11 in T4S is still fundamentally unknown.

VirB4 subunits are large IM proteins with consensus Walker A and B nucleoside triphosphate-binding domains. A combination of experimental studies and computer modeling has yielded a topology model depicting VirB4 as predominantly cytoplasmic with possible periplasmic loops, one near the N-terminus and a second just N-terminal to the Walker A motif. As with VirB11, the contribution VirB4 to machine assembly and function is unknown.

Core subcomplex – VirB6, VirB7, VirB8, VirB9, VirB10

Five VirB proteins are implicated as forming a 'core' transenvelope structure on the basis of phylogenetic relationships, and cell localization and protein–protein interaction data. VirB6 is highly hydrophobic with five predicted transmembrane segments (TMS) and a cytoplasmic C-terminus. A large central periplasmic loop, designated loop P2, is now known to play an important role in substrate translocation (see below). VirB6 interacts with two OM proteins, VirB7 lipoprotein and VirB9, and probably also with the other VirB 'core' subunits. VirB6 exerts stabilizing effects on other VirB subunits, and it colocalizes with VirD4 and the T pilus at the cell poles of *A. tumefaciens*. The available data are consistent with a proposal that VirB6 assembles as a central component of the secretion channel mediating substrate transfer across the IM. The VirB7 lipoprotein forms a disulfide bridge with VirB9, and the heterodimer sorts to the OM where it exerts stabilizing effects on other machine subunits. VirB8 and VirB10 are bitopic IM subunits. Recently, structures of periplasmic fragments of both the subunits were solved by X-ray crystallography. Over its length, VirB10 shares several structural features with TonB, including a small N-terminal cytoplasmic domain, a single TMS, a Pro-rich region, and a region of sequence conservation at the C-terminal end. For TonB, the Pro-rich motif contributes to a rigid, extended structure in the periplasm that might permit simultaneous contacts with partner subunits at the IMs and OMs. Similarly, *A. tumefaciens* VirB10 interacts with the IM subunits VirB8, VirD4, and VirB4, and with the OM-associated VirB7–VirB9 heterodimer. Intriguingly, VirB10 also functionally resembles TonB by linking energy at the IM to the assembly or gating of the T4S channel for substrate translocation (see below).

T pilus subcomplex – VirB2, VirB5, VirB7

The T4S systems involved in conjugation elaborate pili for establishing contact between plasmid-bearing donor cells and recipient cells. Electron microscopy studies have demonstrated the presence of long filaments approximately 10 nm in diameter on the surfaces of *A. tumefaciens* cells induced for expression of the *virB* genes. These filaments are absent from the surfaces of mutant strains defective in expression of one or more of the *virB* genes. Furthermore, the interesting observation was made that cells grown at room temperature rarely possess pili, whereas cells grown at ~19 °C possess these structures in abundance. This finding correlates nicely with previous findings that low temperature stimulates the *virB*-dependent transfer of substrates to plants.

All of the VirB proteins, but not VirD4, are required for the assembly of the T pilus, which is composed of the VirB2 pilin protein. VirB2 bears both sequence and structural similarity to the TraA pilin subunit of the F plasmid and to the TrbB subunit of plasmid RP4. VirB2, like TraA and RP4, is processed from a ~12-kDa propilin to a 7.2-kDa mature protein. Furthermore, both VirB2 and TrbB undergo an unusual head-to-tail cyclization reaction, resulting in a cyclic polypeptide that accumulates in the IM. VirB2 polymerizes as the T pilus, and VirB7 lipoprotein and VirB5 associate at unspecified locations along the T pilus.

Dynamics of T4S System Machine Assembly and Function

Recent studies have begun to describe dynamic properties of T4S systems. These studies have exploited a combination of cytological and biochemical approaches to understand how secretion substrates are recruited to the T4S machine and the route of substrate translocation.

Recruitment of secretion substrates to the VirB/D4 T4S machine

As noted above, ParA/MinD-like VirC1 functions to recruit the processed T-complex to the polar-localized VirD4 SR. Independent of VirC1, however, VirD4 can recruit a protein effector, VirE2, to the cell pole. Whether other protein substrates can also interact directly with VirD4 or require a mediator or coupling factor is not known.

Definition of the T-DNA translocation pathway by TrIP

An assay termed transfer DNA immunoprecipitation (TrIP) was developed to trace the path of a DNA substrate through the T4S channel. TrIP, adapted from the chromatin immuoprecipitation assay, involves formaldehyde treatment of intact cells to cross-link channel subunits to the T-DNA substrate as it exits the cell, disruption of the cells, solubilization of membranes, and immunoprecipitation to recover channel subunits. The presence of T-DNA substrate in the immunoprecipitates is then detected by PCR amplification. With the TrIP assay, it was shown that the substrate forms close contacts with 6 of the 12 VirB/D4 components, VirD4, VirB11, polytopic VirB6, bitopic VirB8, VirB2 pilin, and VirB9. Analyses of various T4S mutants with the TrIP assay enabled formulation of a sequentially and spatially ordered translocation pathway for the T-DNA substrate. This pathway provides the first glimpse of how the T4S channel might be configured across the cell envelope (**Figure 5**). The steps in the pathway are as follows:

- *Substrate recruitment.* The T-DNA substrate binds VirD4 and it does so independently of other VirB proteins, establishing that VirD4 is the T-DNA receptor. A VirD4 Walker A mutant also retains T-DNA as well as protein substrate receptor activity, suggesting that binding of both types of substrates occurs independently of ATP energy.
- *Transfer to the VirB11 hexameric ATPase.* Next, VirD4 transfers the T-DNA substrate to the VirB11 ATPase. This early transfer step also proceeds independently of ATP energy, as deduced by the finding that VirD4 or VirB11 Walker A mutations support substrate transfer. However, VirD4 cannot transfer the substrate to VirB11 in the absence of certain 'core' VirB proteins, suggesting that these core components are important for productive communication between VirD4 and VirB11.

- *Transfer to the integral IM proteins VirB6 and VirB8.* VirB11 delivers the T-DNA substrate to the polytopic VirB6 and bitopic VirB8 proteins. VirB6 mutational studies identified a central periplasmic loop, termed P2, which is important for VirB6 binding to the DNA substrate. Other domains were implicated in regulating subsequent substrate translocation steps. Substrate transfer from VirB11 to VirB6 and VirB8 also require additional 'core' subunits, possibly important for VirB11 binding to these latter channel subunits, as well as the energetic contributions of VirB4, a third ATPase of this secretion system.
- *Transfer to the periplasmic and OM-associated proteins VirB2 and VirB9.* VirB2 and VirB9 comprise the distal end of the T-DNA translocation pathway. As noted above, VirB2 polymerizes as the T pilus. Although it is formally possible that the T-DNA substrate moves through the lumen of the pilus to the plant cell, this probably is not the case because certain mutations block pilus production without affecting substrate translocation. In strains producing the 'uncoupling' mutant proteins, the cellular form of VirB2 is still required for substrate transfer. Thus, VirB2 might be a component of the secretion channel extending through the periplasm and, possibly, the OM. Several T4S subunits, including VirB3, VirB5, and VirB10, are required for this step of substrate transfer, but they do not form detectable interactions with the T-DNA. Therefore, VirB3, VirB5, and VirB10 are probably not channel subunits *per se*, but rather contribute to the structural integrity of the channel.

Energetics of DNA translocation: VirB10, a TonB-like ATP energy sensor subunit

Assembly and function of the conjugation machines requires both proton motive force and ATP energy. In *A. tumefaciens*, the bitopic protein VirB10 interacts with VirD4 and was shown to undergo a structural transition in response to ATP utilization by VirD4 and VirB11. VirB10 also interacts with the OM-associated VirB7–VirB9 heterodimer or multimer by a mechanism requiring ATP energy use by VirD4 and VirB11. Accompanying formation of the transenvelope VirD4–VirB10–VirB7–VirB9 complex, the T-DNA substrate translocates from the IM portion of the secretion channel composed of VirB6 and VirB8 to that in the periplasm composed of VirB2 and VirB9. These findings suggest that VirB10 supplies a function similar to that described for the TonB energy transducer proteins. While TonB senses an IM electrochemical gradient, VirB10 senses IM ATP energy. In both the cases, however, IM energy is converted into a mechanical force required for a latter stage of machine biogenesis. VirB10 might transduce IM energy to mediate formation or opening of a VirB7–VirB9 channel complex to allow passage of the DNA substrate to the cell surface.

Spatial Positioning of the VirB/D4 T4S System

A. tumefaciens cells have been shown to via their cell poles to abiotic and biotic surfaces. As noted above, the VirB/D4 T4S system also localizes at cell poles. In recent studies, six VirB proteins – VirB1, VirB5–VirB7, VirB9, and VirB10 – were shown to depend on production of VirB8 for polar localization, whereas VirB3, VirB4, and VirB11 were found to localize at cell poles independently of VirB8. The VirB4 and VirB11 ATPases are not required for polar targeting of other VirB proteins, and VirB4 and VirB11 Walker A mutants display WT localization patterns, suggesting that nucleation of the VirB proteins at the cell pole does not require ATP energy. At this time, therefore, at least three protein complexes have been shown to localize at cell poles independently of each other: (1) the relaxosome bound at T-DNA border sequences that consists of VirD1, VirD2, VirC1, and VirC2; (2) the VirD4 SR; and (3) the VirB channel complex. Adding to this picture, the Ti plasmid itself localizes at or near the cell poles of *A. tumefaciens* vegetative cells. It will be interesting in future work to identify the underlying molecular basis for polar targeting of these various protein complexes, and also to understand how these machine complexes coordinate their activities in space and time to mediate translocation of T-DNA and protein substrates to target cells.

Substrate Transfer Through the Plant Cell

The delivery of T-DNA and protein substrates to plant cells requires productive contact between *A. tumefaciens* and a susceptible plant cell. *A. tumefaciens* commonly infects plants at wound sites, giving rise to a widely held view that wounding establishes important preconditions for infection. During wounding, plants release cell wall constituents and such molecules are potent *vir* gene inducer molecules. Wounding potentially also creates portals of entry through damaged cell walls, and stimulates cell replication and division reactions considered to be important for T-DNA integration. However, it is now known that *A. tumefaciens* can deliver T-DNA to unwounded plant tissues, dispelling the notion that wounding is an essential prerequisite for transformation. The most visible manifestation of *A. tumefaciens* transformation is the production of plant tumors, yet transformation of unwounded tissues typically does not incite tumor formation. Many transformation events, therefore, might be phenotypically silent, raising the intriguing possibility that *A. tumefaciens* actually has a much broader host range and can infect different plant cell types than reported previously.

Attachment to Plant Cells

A. tumefaciens must bind plant cells to deliver T-DNA across the plant plasma membrane. Recent evidence indicates that there are at least two binding events that may act sequentially or in tandem. The first is encoded by chromosomal loci and occurs even in the absence of the Ti plasmid genes. This binding event directs bacterial binding to many plant cells independently of whether or not the bacterium is competent for exporting T-DNA or the given plant cell is competent for the receipt of T-DNA. The second binding event is mediated by the *virB*-encoded T pilus.

Binding via the chromosomally encoded attachment loci is a two-step process in which bacteria first attach loosely and nonspecifically to the plant cell surface. This is a nonsaturable and aggregation-like mode of interaction reversible by washing with a buffered salt solution. Next, the bacteria attach more specifically in a tight and saturable interaction that is resistant to washing. A series of genes designated attachment (*att*) genes were implicated in mediating the latter mode of attachment, though this has been questioned because the *att* genes reside on a 542 kb plasmid, pAtC58, which has been shown to be dispensable for virulence. Another set of genes designated as *cel* direct the synthesis of cellulose fibrils that emanate from the bacterial cell surface. These fibrils are implicated in attachment of *A. tumefaciens* to specific sites on the plant cell surface. Binding is saturable, suggestive of a limited number of attachment sites on the plant cell, and binding of virulent strains can also be prevented by the attachment of avirulent strains.

Efficient attachment of bacteria to plant cells also requires the products of three chromosomal loci, *chvA, chvB*, and *exoC* (*pscA*). All three loci are involved in synthesis of transport of a cyclic β-1,2 glucan molecule. Mutations in these genes are pleiotropic, suggesting that β-1,2 glucan synthesis is important for the overall physiology of *A. tumefaciens*. Periplasmic β-1,2 glucan plays a role in equalizing the osmotic pressure between the inside and outside of the cell. It has been proposed that loss of this form of glucan may indirectly disrupt virulence by reducing the activity or function of cell surface proteins. Interestingly, *chv* mutants accumulate low levels of VirB10, one of the proposed components of the T-complex transport system (see 'The VirB/D4 System, a Member of the Type IV Secretion Family'), suggesting that β-1,2 glucan might influence T-DNA export across the bacterial envelope by contributing to transporter assembly.

Recent genomic studies have begun to identify possible plant proteins involved in attachment. A collection of mutations in *Arabidopsis thaliana* were generated that render the host plant recalcitrant to *Agrobacterium* transformation (*rat* mutants). Some of these mutations disrupt attachment of *Agrobacterium* and thus might map to surface receptors. One such mutation is in the promoter of a gene encoding arabinogalactan, a probable cell wall constituent. Another candidate receptor is a vitronectin-like protein found in detergent extracts of plant cell walls. Attachment-proficient *A. tumefaciens* cells bind radioactive vitronection, whereas attachment-deficient cells do not bind this molecule. Intriguingly, human vitronectin and antivitronectin antibodies both inhibit the binding of *A. tumefaciens* to plant cells. Yet other candidate receptors identified to date include a rhicadhesin-binding protein, a cellulose synthase-like protein, and several proteins shown to bind the VirB2 pilin protein. These proteins, designated VirB2-interacting proteins (VBTs), might mediate binding of the T pilus to the plant cell surface.

Substrate Movement Through the Plant Cytosol and Integration into the Host Genome

The VirD2–T-strand complex is only one of several substrates delivered to plant cells through the VirB/D4 T4S system. The others identified to date include the VirE2, VirE3, VirF, and VirD5 proteins. VirE2 is a single-stranded DNA-binding protein required for transformation, whereas the other translocated substrates function to enhance the efficiency of transformation. VirE2 is exported separately from the VirD2–T-strand particle, but upon transfer to the plant VirE2 binds cooperatively to the T-strand, forming a VirD2–T-strand–VirE2 particle termed the T-complex. The T-complex, composed of a ~20-kb T-strand capped at its 5′ end with a 60-kDa endonuclease and an estimated 600 molecules of VirE2 along its length, is a large nucleoprotein complex of an estimated size of 50×106 Da. Evidence exists that VirD2 and VirE2 protect the T-strand from plant nucleases and also facilitate T-complex movement along a microtubule network. VIP1, a protein shown to interact with VirE2, is postulated to function as a molecular link between the T-complex and microtubule track system. VirD2 also has been shown to interact with several members of a family of proteins termed cyclophilins. The postulated role for cyclophilins in this interaction is to supply a chaperone function at some stage during T-complex trafficking to the nucleus. *A. tumefaciens* has been demonstrated to transport DNA to representatives of prokaryotes, yeasts, and plants. Cyclophilins are ubiquitous proteins found in all of these cell types and, therefore, may be of general importance for *A. tumefaciens*-mediated DNA transfer.

Additionally, both VirD2 and VirE2 carry nuclear localization sequences (NLS) that contribute to delivery of the T-complex to the nuclear pore. VirD2 was shown to interact with AtKAPa, a member of a conserved family of importin/karyopherin proteins that are known to bind NLS and mediate nuclear import. Correspondingly, VIP1 is postulated to mediate nuclear import of VirE2. These different plant proteins, thus, might act synergistically or redundantly to mediate movement of the T-complex through the plant cytoplasm and nuclear pore. Interestingly, VirE3, another translocated substrate, mimics the function of VIP1 in mediating VirE2 nuclear import, possibly explaining how *A. tumefaciens* can transform nonplant species such as yeast and human cells that lack VIP1.

Once inside the nucleus, the T-complex must be delivered to its site of integration in the host chromatin. Plant proteins, including CAK2M and TATA box-binding proteins that bind VirD2, VIP1 that binds VirE2, and core histones that bind VIP2, may be important for chromatin targeting of the T-complex. T-DNA integrates into the plant nuclear genome by a process termed 'illegitimate' recombination. According to a current model, T-DNA invades at nicks or gaps in the plant genome possibly generated as a consequence of active DNA replication. The invading ends of the single-stranded T-DNA are proposed to anneal via short regions of homology to the unnicked strand of the plant DNA. Once the ends of T-DNA are ligated to the target ends of plant DNA, the second strand of the T-DNA is replicated and annealed to the opposite strand of the plant DNA. Both VirD2 and VirE2 have been implicated in contributing to the T-DNA integration step, but the molecular details of this reaction are not known. However, another translocated substrate, VirF, has been shown to possess an F-box domain and interact with several members of the ASK protein family, which are plant homologues of yeast Skp1 proteins. F-box and Skp1 are conserved components of E2 ubiquitin ligases that mediate protein destabilization. VirF was shown to destabilize a VIP1–VirE2 complex and thus might play a role in uncoating the T-DNA prior to or during integration into the host genome. Clearly, movement of T-complexes and integration of T-DNA into the plant genome is a complex multistep process involving specific binding of plant factors with bacterial effector proteins. However, note that all characterized effectors identified to date participate in some way to the movement of T-DNA through the plant cell or its integration into the plant genome. Whether the armament of translocated effectors includes proteins whose functions are unrelated to T-DNA movement and instead involved in disruption of plant physiological processes to promote the overall infection process is an interesting question for further study.

Agrobacterium Host Range and Genetic Engineering

One of the most appealing features of the *A. tumefaciens* DNA transfer system for genetic engineering is its extremely broad host range. *Agrobacterium* has long been known to transform a wide range of gymnosperms and dicotyledonous plant species of agricultural importance. Additionally, during the past two decades protocols have been developed for transformation of monocotyledonous plant species including rice, wheat, and maize. So far, most of the effort in developing these transformation protocols has been directed toward improvement of crop traits. Increasingly, however, the *A. tumefaciens* gene delivery system is being used to (1) isolate and characterize novel plant genes through T-DNA tagging, (2) deliver foreign DNA to specific sites in the plant genome, and (3) genetically engineer nonplant organisms. Intriguingly, *Agrobacterium* is now known to transform many nonplant species including other prokaryotes, yeast, and many other fungi, and human cells. And now, with the discovery that other alphaproteobacterial species including *Rhizobium* sp. NGR234, *S. meliloti*, and *M. loti* also deliver DNA substrates to plant target cells, the potential exists that the host range of eukaryotic cell types transformable by bacteria can be broadened even further.

Nuts and Bolts of Genetic Engineering

A. tumefaciens is readily manipulated such that plasmids carrying foreign genes of interest are easily introduced into appropriate bacterial strains for delivery to plants. Typically, strains used for gene delivery are 'disarmed', that is, deleted of oncogenic T-DNA, but still harboring intact Ti plasmid and chromosomal *vir* genes. Foreign genes destined for delivery to plants are generally cloned onto a plasmid that carries a single T-DNA border sequence or two T-DNA border sequences that flank various restriction sites for cloning as well as an antibiotic resistance gene to select for transformed plant cells. If the plasmid carries a single border sequence, the entire plasmid is delivered to plants, and surprisingly *A. tumefaciens* is capable of delivering in excess of 180-kb of DNA to plants. If the plasmid carries two border sequences, only the DNA bounded by T-DNA borders is delivered to plants. The frequency of stable transformation is often very high, well-exceeding frequencies achieved by other gene delivery methods. For example, cocultivation of *A. tumefaciens* with regenerating protoplasts of certain plant species can result in transformation of up to one half of the protoplasts.

However, with protoplast transformation there is often a significant reduction in the number of transgenic, fertile plants recovered during selective regeneration of transformed protoplasts. For certain species, protoplasts can be transformed but are recalcitrant to regeneration into intact plants. Consequently, other transformation methods have relied on transformation of plant tissues such as excised leaves or root sections. In the case of monocot species such as maize, immature embryos are the preferred starting material for *A. tumefaciens*-mediated DNA transfer. For rice, success has been achieved with callus tissue induced from immature embryos. Additional factors such as plant genotype, the type and age of plant tissue, the kinds of vectors and bacterial strains, and the types of selectable genes delivered to plant cells all influence the transformation efficiencies. For rice and corn, most of these parameters have been optimized so that now the delivery of foreign DNA to these crop plants is a routine technique.

In addition to the need to identify transformable and regenerable plant tissues, a number of varieties of a given species often need to be screened to identify the susceptible varieties. A large variation in transformation efficiencies is often observed depending on which cell line is being tested. This underscores the notion that interkingdom DNA transfer is a complex process dependent on a genetic interplay between *A. tumefaciens* and host cells. Fortunately, many of the agronomically important species are readily transformable, but further efforts are needed to overcome present obstacles impeding efficient transformation of other species of interest.

T-DNA Tagging

A. tumefaciens is increasingly used to characterize and isolate novel plant genes by an approach termed T-DNA tagging. Several variations to this methodology exist depending on the desired goals. For example, because insertions are generally randomly distributed throughout the plant genome, T-DNA is widely used today as a mutagen for isolating plant genes with novel phenotypes. If the mutagenic T-DNA carries a bacterial origin of replication, the mutated gene of interest can easily be recovered in bacteria by suitable molecular techniques. Further, if the T-DNA is engineered to carry a selectable or scorable gene near one of its ends, insertion downstream of a plant promoter will permit characterization of promoter activity. Conversely, if the T-DNA is engineered to carry on outward reading promoter, insertion can result in a modulation of gene expression with potentially interesting phenotypic consequences. Finally, the discovery that *A. tumefaciens* can transform fungal species of interest means that all approaches developed for plants now can be applied to the characterization of fungi.

Homologous or Site-Specific Recombination

Although random T-DNA insertion is a boon to investigators interested in characterizing plant or fungal genes, it is an undesired event for plant genetic engineering. In addition to the potential result that T-DNA will insert into an essential gene, insertion is often accompanied by rearrangements of flanking sequences, which further enhances the chances that the insertion will have undesired consequences. Ideally, T-DNA could be delivered to a restricted number of sites in the plant genome. Recent progress toward this goal has involved the use of the bacteriophage P1 Cre/lox system for site-specific integration in the plant genome. The Cre site-specific recombinase catalyzes strand exchange between two lox sites, which, for P1, results in circularization of the P1 genome upon infection of bacterial cells. For directed T-DNA insertion, both the plant and the T-DNA are engineered to carry lox sequences and the plant is also engineered to express the Cre protein. Upon entry of T-DNA into the plant cell, Cre was shown to catalyze the site-specific integration of T-DNA at the plant lox site. The frequency of directed insertion events is low compared to random insertion events, but further manipulation of this system should enhance its general applicability.

Gene Transfer to Yeast and Fungi

The successful transfer of DNA to yeast depends on the presence of stabilizing sequences such as a yeast origin of replication sequence or a telomere, or regions of homology between the transferred DNA and the yeast genome for integration by homologous recombination. When the T-DNA lacks any extensive regions of homology with the *Saccharomyces cerevisiae* genome, it integrates at random positions by illegitimate recombination reminiscent of T-DNA integration in plants. The transformation of filamentous fungi with *A. tumefaciens* is an exciting advancement. *A. tumefaciens* was shown to efficiently deliver DNA to fungal protoplasts as well as fungal conidia and hyphal tissue. This discovery extends well beyond academic interest because the simplicity and high efficiency make this gene delivery system an extremely useful tool for the genetic manipulation and characterization of fungi. This DNA transfer system is especially valuable for species such as the mushroom *Agaricus bisporus* that are recalcitrant to transformation by other methods. It is also of interest to consider that both *A. tumefaciens* and many fungal species exist in the same soil environment, raising the possibility that *A. tumefaciens*-mediated gene transfer to fungi may not be restricted solely to the laboratory bench.

Conclusions

The early discovery that the oncogenes can be excised from T-DNA and replaced with genes of interest paved the way for the fast-growing industry of plant genetic engineering. Today, a large amount of information has been assembled about the *A. tumefaciens* infection process. This information has been used to successfully manipulate the T-DNA transfer system both to enhance its efficiency and to broaden the range of transformable plants and other organisms. Furthermore, this information has often established a conceptual framework for initiating or extending studies of other pathogenic and symbiotic relationships. The discovery that secreted chemical signals comprise the words for a dynamic dialog between *A. tumefaciens* and plant cells as well as other *A. tumefaciens* cells has stimulated a global effort to identify extracellular signals and characterize the cognate signal transduction systems in many bacterial systems. The discovery of T-DNA transport itself supplied a mechanistic explanation for how horizontal gene transfer impacts the evolution of genomes of higher organisms. This discovery also established a precedent for interkingdom transport of virulence factors by bacterial pathogens. Indeed, just in the last decade, studies have revealed that numerous pathogens employ interkingdom transport to deliver a wide array of effector proteins to plant and animal hosts. Interkingdom macromolecular translocation is mediated either by the T4S systems, which are ancestrally related to conjugation systems, or by the T3S systems, ancestrally related to flagellar systems. Both T3S and T4S systems translocate substrates via processes dependent on cell-to-cell contact and, in some cases, elaboration of an extracellular filament or pilus. For the future, it is clear that studies of all the various aspects of the *A. tumefaciens* infection process will continue to spawn new applications for this novel DNA transfer system and yield new insights about the evolution and function of pathogenic mechanisms that are broadly distributed in nature.

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Amino Acid Production

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Glossary

carriers/transporters Membrane proteins that function to transport substances into or out of the cell through the cytoplasmic membrane.

fermenter A large growth vessel used to culture microorganisms on a large scale for the production of commercially valuable products.

immobilized cell A cell attached to a solid support over which substrate is passed and is converted into product.

metabolic engineering The improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the application of recombinant DNA technology.

regulation Processes that control the activities or synthesis of enzymes.

selection Placing organisms under conditions where the growth of those with a particular genotype is favored.

Abbreviations

AEC	analogue aminoethyl-L-cysteine
CSL	corn steep liquor
DO	dissolved oxygen
GA	glutaraldehyde
HA	hexamethylene diamine
MSG	monosodium glutamate
OUR	oxygen uptake rate
PGDH	3P-glycerate dehydrogenase
SHMT	serine hydroxymethyltransferase
THF	tetrahydrofolate derivative

Introduction

Amino acids are simple organic compounds that contain one or more amino groups and one or more carboxyl groups. They are the building blocks of peptides, proteins, and components of other complex polymers like the cell wall. There are 20 protein-forming amino acids, all of which, except glycine, are optically active and occur as L-enantiomers. Eight of these protein-forming L-amino acids are essential for mammals. There are large demands for amino acids in the areas of food and feed additives and drug manufacturing. In medicine, amino acids are used for infusions and as therapeutic agents. Amino acid derivatives are also used in the chemical industry, such as in synthetic leathers, surface-active agents, fungicides, and pesticides. It is estimated that in 2004, a volume of 56% of the total amino acid market was occupied by the so-called feed amino acids L-lysine, DL-methionine, L-threonine, and L-tryptophan. At 34%, the share of the food sector is also substantial and is essentially determined by three amino acids L-glutamate, in the form of the flavor enhancer monosodium glutamate (MSG), and L-aspartate and L-phenylalanine, the latter two used as starting materials for the peptide sweetener L-aspartyl L-phenylalanyl methyl ester (aspartame). The total amino acids manufactured represent a value of roughly US$5000 million, and thus trail behind antibiotics made with microorganisms. The market is growing steadily by about 5–10% per year. The production methods to date are (1) microbial production, (2) protein hydrolysis, (3) chemical synthesis, and (4) enzymatic synthesis. Whereas chemical synthesis produces a racemic mixture, which may require additional resolution, the other procedures give rise to optically pure L-amino acids.

Microbial Production

Development of Amino Acid-Overproducing Strains

Many bacteria are capable of growing on a simple mineral salt medium containing ammonium, phosphate, further salts, and glucose as the carbon and energy source. These bacteria are able to synthesize all the compounds necessary for the living cell from these simple nutrients. The dry matter of the bacterial cell consists of about 60% protein, 20% nucleic acids, 10% carbohydrates, and 10% fat. Therefore, the cell must be able to synthesize amino acids rapidly and efficiently. However, as a rule, only as much of the various amino acids as required for growth are synthesized in the bacterial cell, that is, normally, the bacteria do not overproduce and excrete amino acids into the culture medium. The reason is that bacteria have a number of sophisticated regulatory mechanisms, like repression and feedback inhibition through end products, that economically control the overproduction of metabolites.

Thus, classical mutagenesis has been applied to obtain mutants that are able to overproduce a specific amino acid in large amounts. Regulatory strains were obtained by selecting mutants that are resistant to amino acid analogues. Some of the commonly used amino acid analogues – lysine, threonine, and tryptophan – are given in **Table 1**. Amino acid analogue resistance may be because of derepression of the enzymes involved in the biosynthesis of amino acids or the elimination of the allosteric control of biosynthetic key enzymes. Furthermore, the amount of amino acids synthesized via a branched pathway can be significantly increased by selecting strains auxothrophic for the competing branch. Mutagenesis and selection is one of the most important techniques for the development of amino acid-overproducing microorganisms, and such mutants are used in developing superior production strains.

The current repertoire of strain development includes the following:

1. Identification of mutations in classical strains and their introduction via recombinant DNA techniques into a wild type, thus rebuilding the producer. (Because of undirected mutagenesis, mutations that are disadvantageous might have accumulated, thus reducing growth or speed of sugar conversion.)
2. Combination of beneficial mutations of different strains.
3. Gene overexpression to overcome limiting steps.
4. Identification of genes by global microarray DNA analysis whose expression correlates with favorable producer strains or fermentations, and the subsequent controlled expression of such genes by genetic means.
5. Application of intracellular flux quantifications using ^{13}C-labeled substrate and ^{13}C-NMR.
6. Manipulation of export activity to increase the export of amino acids.

The improvement in cellular activities by directing the enzymatic, transport, and regulatory functions of the cell by the use of recombinant DNA technology is now a standard method for obtaining highly productive amino acid-producing strains, often based on strains derived by undirected mutagenesis.

L-Glutamic Acid

Following the increasing demand for MSG as a flavoring agent in the mid-1950s, a bacterium that excreted large quantities of the amino acid L-glutamic acid into the culture medium was isolated in Japan. This bacterium, *Corynebacterium glutamicum*, is a short, aerobic, Gram-positive rod capable of growing on a simple mineral salt medium with glucose, provided that biotin is also added. A recent monograph collected a great number of the existing features of *C. glutamicum*, leading to amino acid production. The production of L-glutamic acid by *C. glutamicum* is maximal at a critical biotin concentration of 0.5 $\mu g\, g^{-1}$ of dry cells, which is suboptimal for growth. Despite being effectively produced for over 50 years in amounts of 1.8 million tons per year, the molecular features resulting in L-glutamate excretion are still poorly understood. However, besides biotin limitation, a surprisingly large variety of other treatments also result in L-glutamate excretion, for example, addition of selected

Table 1 Analogues of amino acids used for the selection of L-lysine-, L-threonine-, or L-tryptophan-overproducing strains

Lysine	*Threonine*	*Tryptophan*
S-(2-aminoethyl)-L-cysteine (AEC)	α-Amino-β-hydroxyvaleric acid	5-Methyltryptophan
4-Oxalysine	β-Hydroxyleucine	4-Methyltryptophan
L-lysine hydroxamate	Norleucine	6-Methyltryptophan
2,6-Diamino-4-hexenoic acid	Aminohydroxyvaleric acid	5-Fluorotryptophan
2,6-Diamino-4-hexenoic acid	N-Lauryl leucine	6-Fluorotryptophan
ε-C-Methoylysine (2,6-diamino-heptanoic acid)	Norvaline	DL-7-Azatryptophan
δ-Hydroxylysine	N-2-Thienoylmethionine	2-Azatryptophan
α-Chlorocaprolactam	2-Amino-3-methylthiobutyric acid	3-Inolacrylic acid
Trans-4,5-dehydrolysine	2-Amino-3-hydroxyhexanoic acid	Indolmycin

detergents like Tween-40, addition of penicillin, use of fatty acid auxotrophic strains, or addition of ethambutol-inhibiting arabinogalactan synthesis. Interestingly, all these conditions have more or less in common to target the cell wall or cell membrane. Moreover, it is known that under L-glutamate-producing conditions ketoglutarate dehydrogenase activity is low. Only recently an exciting discovery was made giving at a blow an idea how all these different characteristics might be linked. It was found that *C. glutamicum* possesses a regulatory OdhI protein, together with a eukaryotic-type serine/threonine protein kinase (PknG) and a phosphatase (Ppp), the latter two controlling the phosphorylation status of OdhI. Unphosphorylated OdhI inhibits ketoglutarate dehydrogenase activity and the OdhI protein was shown to be necessary for high L-glutamate production. Importantly, Ppp is apparently membrane bound, as is a second serine/threonine protein kinase likely to additionally control the phosphorylation status of OdhI. Also, the genes controlling the phosphorylation status are adjacent to genes of cell wall synthesis and cell division. Thus, a disordered cell wall or cell membrane might be sensed by the system and might inhibit ketoglutarate dehydrogenase activity. As a consequence, α-oxoglutarate is not further metabolized in the citric acid cycle, but instead is converted into L-glutamic acid, by reductive amination catalyzed by the NADP-dependent glutamate dehydrogenase present in *C. glutamicum.*

In *C. glutamicum*, glucose is mainly metabolized via the glycolytic pathway into C_3 and C_2 fragments. Oxaloacetate is formed via the phosphoenolpyruvate carboxylase and the pyruvate carboxylase (**Figure 1**). Thus, *C. glutamicum* has two anaplerotic reactions for the conversion of the C_3 intermediates into oxaloacetate. These reactions are important to always have sufficient oxalaoacetate available for condensation with acetyl-CoA and conversion of citrate to α-oxoglutarate. The overall reaction for L-glutamic acid production from D-glucose is as follows:

$$C_6H_{12}O_6 + NH_3 + 3NAD^+ \rightarrow C_5H_9O_4N + 3NADH + 3H^+ + CO_2$$

Thus, the theoretical maximal yield is $1\,\mathrm{mol\,l^{-1}}$ of L-glutamic acid per mole of glucose metabolized. This represents a 100% molar conversion or 81.7% weight conversion of D-glucose to L-glutamic acid.

The L-glutamic acid production is carried out in stirred fermenters up to the size of $500\,\mathrm{m^3}$. Provisions for cooling, dissolved oxygen measurement, pH measurement, and control (usually with ammonium) are required. A temperature between 30 °C and 35 °C and a pH between 7.0 and 8.0 are optimal. The oxygen transfer rate is fairly critical: a deficiency leads to poor glutamate yields, with lactic and succinic acid being formed instead, while an excess causes accumulation of α-oxoglutaric acid. The yield of L-glutamic acid obtained after 2–3 days of incubation is in the order of 60–70% (by weight) of the sugar supplied, and the final concentration is approximately $150\,\mathrm{g\,l^{-1}}$.

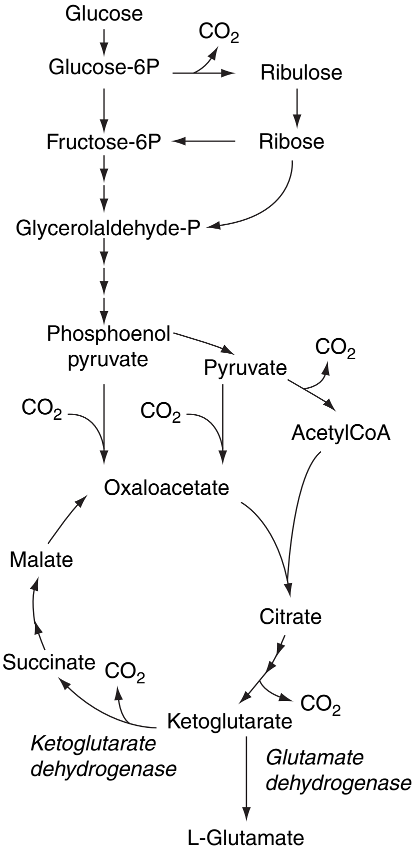


Figure 1 Biosynthesis of L-glutamic acid in *Corynebacterium glutamicum* using glucose as the carbon source.

L-Lysine

L-lysine, an amino acid essential for human and animal nutrition, is mainly used as a feed supplement as it is present in most plant proteins only in low concentrations. At present, approximately 700 000 tons per year of L-lysine is produced using mutant strains of *C. glutamicum.* The wild type does not secrete L-lysine into the culture medium. However, excellent high-yield production strains were developed by mutation and by selection for antimetabolite resistance together with modern recombinant DNA techniques.

The pathway for the biosynthesis of L-lysine in *C. glutamicum* is illustrated in **Figure 2**. A remarkable feature of *C. glutamicum* is its split pathway for the synthesis of L-lysine. At the level of L-2,3,4,5-tetrahydrodipicolinate there are two pathways for the conversion of this precursor into DL-2,6-diaminopimelate. The first enzyme in L-lysine biosynthesis, aspartokinase, is regulated by concerted

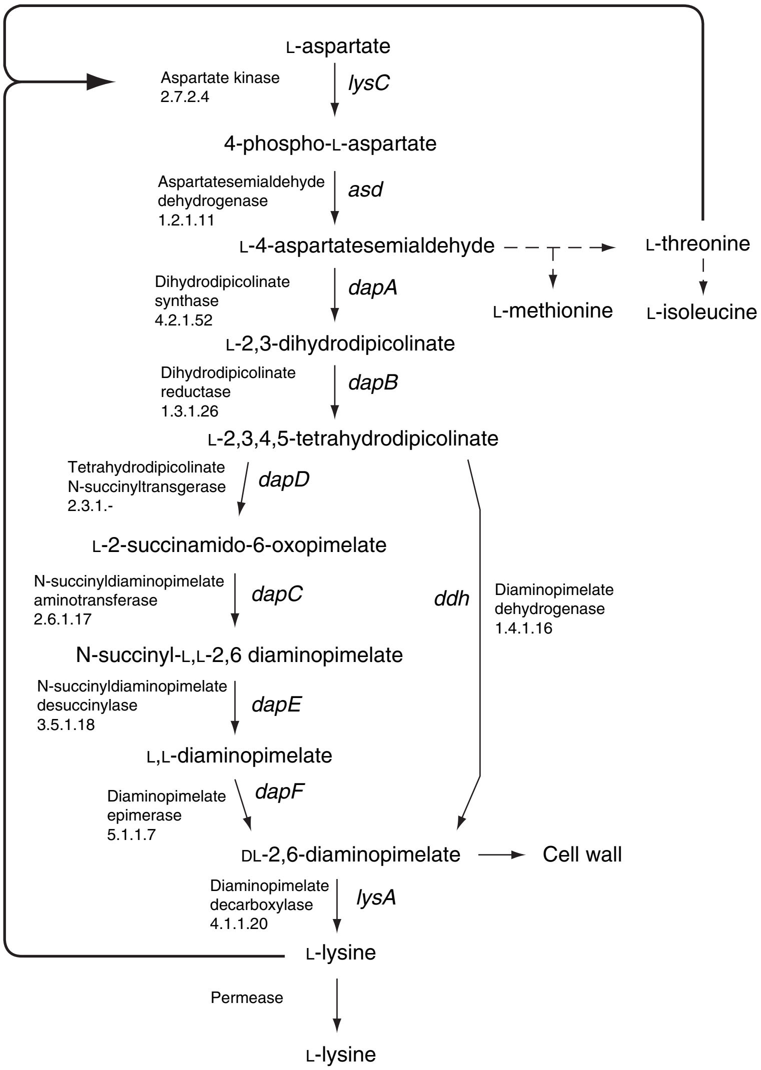

L-aspartate
Aspartate kinase
2.7.2.4
lysC
4-phospho-L-aspartate
Aspartatesemialdehyde
dehydrogenase
1.2.1.11
asd
L-4-aspartatesemialdehyde
L-threonine
L-methionine
L-isoleucine
Dihydrodipicolinate
synthase
4.2.1.52
dapA
L-2,3-dihydrodipicolinate
Dihydrodipicolinate
reductase
1.3.1.26
dapB
L-2,3,4,5-tetrahydrodipicolinate
Tetrahydrodipicolinate
N-succinyltransgerase
2.3.1.-
dapD
L-2-succinamido-6-oxopimelate
N-succinyldiaminopimelate
aminotransferase
2.6.1.17
dapC
ddh
Diaminopimelate
dehydrogenase
1.4.1.16
N-succinyl-L,L-2,6 diaminopimelate
N-succinyldiaminopimelate
desuccinylase
3.5.1.18
dapE
L,L-diaminopimelate
Diaminopimelate
epimerase
5.1.1.7
dapF
DL-2,6-diaminopimelate
Cell wall
Diaminopimelate
decarboxylase
4.1.1.20
lysA
L-lysine
Permease
L-lysine

Figure 2 The split pathway of L-lysine biosynthesis and its regulation in *Corynebacterium glutamicum*; thick arrows = feedback inhibition.

feedback inhibition by L-threonine and L-lysine. Hence, a homoserine auxotroph or a threonine and methionine double auxotroph of *C. glutamicum* diminishes the intracellular pool of threonine, reduces its marked feedback inhibitory effect on aspartokinase, and promotes lysine overproduction (15–30 g l^{-1}). Another effective technique for obtaining L-lysine-producing strains is the selection of regulatory mutants. Growth of *C. glutamicum* is inhibited by the L-lysine analogue aminoethyl-L-cysteine (AEC). This inhibition is markedly enhanced by L-threonine, but reversed by L-lysine. This implies that AEC behaves as a false feedback inhibitor of aspartokinase. Some mutants, which are capable of growing in the presence of both AEC and L-threonine, contain an aspartokinase that is insensitive to the concerted feedback inhibition; therefore, L-lysine is overproduced (30–35 g l^{-1}). L-Aspartate as the precursor of L-lysine synthesis is formed from oxaloacetate by the anaplerotic reaction of phosphoenolpyruvate and pyruvate carboxylation (**Figure 1**). By combined overexpression of aspartokinase and dihydrodipicolinate synthase, L-lysine production can be further increased by 10%. Also, the ample supply of NADPH is important, which is achieved by an increased flux via the pentose phosphate shunt.

In addition to all the steps considered so far, the active export of L-lysine into the culture medium is important. Export is mediated by the specific exporter LysE, a small membrane protein of 25.4 kDa, with five transmembrane spanning helices (**Figure 3**). Expression of this exporter is controlled by the LysR-type transcriptional regulator LysG. At elevated intracellular L-lysine concentrations of 35 mmol l^{-1}, LysG binds L-lysine and drives expression of the exporter gene to result in an about 20-fold increased induction. As a consequence, effective export

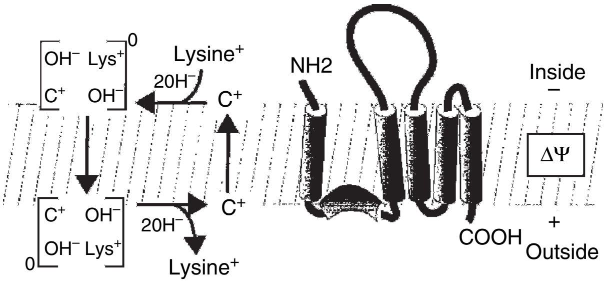


Figure 3 Structure of the L-lysine exporter and the putative mechanism of L-lysine excretion in *Corynebacterium glutamicum*.

occurs, enabling high export rates of up to 7 nmol min^{-1} mg(dry weight)$^{-1}$. The components driving L-lysine export are the electrochemical proton potential and the chemical concentration gradient of L-lysine. A formal description of the energetic steps during translocation involves symport of the positively charged L-lysine with two OH^- (**Figure 3**). For the substrate translocation step the pH gradient and the L-lysine gradient are important, whereas reorientation of the carrier involves the membrane potential. This transporter is a genetic and biochemical system, well designed for excretion purposes:

- It is only induced at eleveated intracellular L-lysine concentrations of about 40 mmol l^{-1}.
- It has a high K_m value for L-lysine (20 mmol l^{-1}) at the internal (cytoplasmic) side, thus preventing efflux under low internal lysine concentration.

In fed-batch culture, L-lysine production strains are able to reach, under the optimized culture conditions, final concentration of at least 170 g l^{-1} L-lysine, and the conversion rate relative to sugar used is about 50%. A typical L-lysine production curve is shown in **Figure 4**; together with sugar ammonium also has to be fed. The convential route of lysine downstream processing is characterized by:

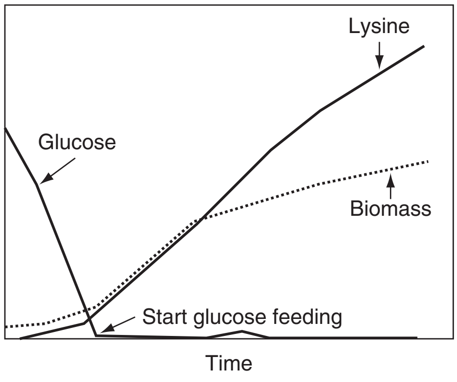


Figure 4 Production of L-lysine with a mutant strain of *Corynebacterium glutamicum*; glucose is fed together with ammonium.

- removal of the bacterial cells from the fermentation broth by separation or ultrafiltration,
- absorbtion and then collection of lysine in an ion exchange step, and
- crystallization or spray drying as L-lysine hydrochloride.

An alternative process consists of biomass separation, concentration of the fermentation solution, and filtration of precipitated salts. The liquid product contains up to 50% of a L-lysine base that is stable enough to be marketed.

Recently, a new concept for lysine production was introduced, in which the entire L-lysine-containing fermentation broth is spray dried and granulated to yield a feed-grade product that contains L-lysine sulfate corresponding to at least 60% of L-lysine hydrochloride. Waste products usually present in conventional L-lysine hydrochloride manufacture are thus avoided in this process.

In 2005, the price for L-lysine × HCl feed-grade was approximately US$2 per kg. With the benefits provided by modern techniques, such as genetic engineering, and with the potential of fermentation technology, additional improvements in the L-lysine process should be realized. L-lysine will continue to be the most attractive feed additive produced by fermentation.

L-Threonine

Until 1986, L-threonine was used mainly for medical purposes, in amino acid infusion solutions, and in nutrients. It was manufactured by extraction of protein hydrolyzates or by fermentation using mutants of coryneform bacteria in amounts of some hundred tons per year, worldwide. The production strains were developed by empirical classical breeding, introduction of auxotrophies, and resistance to threonine analogues such as α-amino-β-hydroxy-valerate, and reached product concentrations up to 20 g l^{-1}. Although the pathway of L-threonine biosynthesis in *Escherichia coli* is much more regulated compared to that in *C. glutamicum*, *E. coli* strains have proved to be superior to *C. glutamicum*. The reason is that *E. coli* has much more effective excretion systems for L-threonine and related amino acids, which are not present in *C. glutamicum*. *E. coli* strains with excellent threonine yield and productivity are now available.

In *E. coli*, the regulation of the L-aspartate-derived amino acids involves several isoenzymes. As shown in **Figure 5**, the phosphorylation of L-aspartate, the first reaction in the biosynthetic pathway of L-threonine, is catalyzed by three different aspartokinases. One of these isoenzymes is inhibited by L-threonine and its synthesis is repressed by L-threonine and L-isoleucine. The second aspartokinase

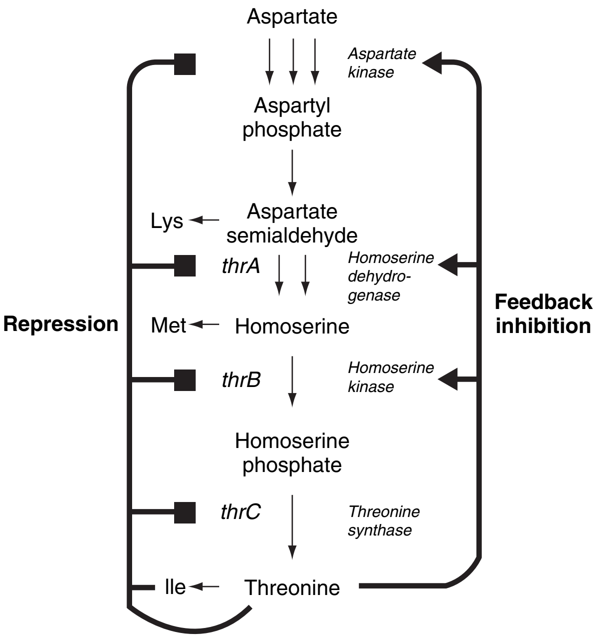


Figure 5 Regulation of L-threonine biosynthesis in *Escherichia coli*. Only the regulation by L-threonine and L-isoleucine is shown.

isoenzyme is repressed by L-methionine, and the third one is inhibited and repressed by L-lysine. Threonine biosynthesis occurs by the conversion of aspartate-β-semialdehyde in three enzymatic steps that are encoded in *E. coli* by the *thrABC* operon. The *thr*A gene encodes a bifunctional enzyme with aspartokinase and homoserine dehydrogenase activities. The *thr* operon is under the control of a single promoter, which is bivalently repressed by L-threonine plus L-isoleucine. Additionally, L-threonine synthesis is regulated by feedback inhibition of the homoserine dehydrogenase and homoserine kinase. L-threonine ranks third in production volume among the biotechnologically produced amino acids behind L-lysine and L-glutamic acid.

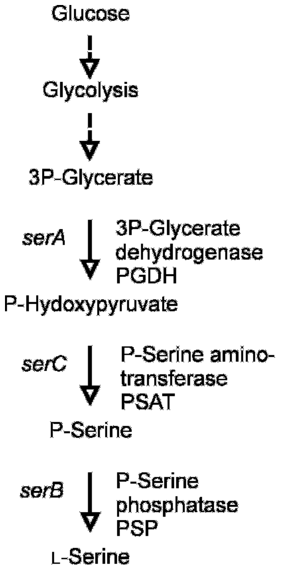
Based on the synthesis pathway, there is a clear focus on two major targets for the design of L-threonine-overproducing strains, that is, the prevention of L-isoleucine formation and stable high-level expression of *thrABC* operon. Therefore, in one of the initial steps of strain development, chromosomal mutations were introduced to result in an isoleucine leaky strain, which requires L-isoleucine only at low L-threonine concentrations; however, at high L-threonine concentrations, growth is independent of the addition of L-isoleucine. This mutation has several advantageous consequences: first, it prevents an excess formation of the undesired by-product L-isoleucine; second, it prevents the L-isoleucine-dependent premature termination of the *thrABC* transcription. Furthermore, the isoleucine leaky mutation has a positive selection effect on all the cells containing the plasmid with the threonine operon. To obtain very high activities of the *thrABC*-encoding enzymes, this operon was cloned from a strain in which the aspartokinase and homoserine dehydrogenase activities are resistant to L-threonine inhibition and was overexpressed. To prevent the degradation of L-threonine the gene *tdh*, which encodes threonine dehydrogenase, was inactivated.

By continuous sugar feeding of such an *E. coli* strain L-threonine concentrations of more than $80\,\mathrm{g\,l^{-1}}$ are obtained with a conversion yield of about 60%. The recovery of feed-grade L-threonine is rather simple: after the cell mass has been removed from the culture broth by ultrafiltration, the filtrate is concentrated and the amino acid is isolated by crystallization. Currently, L-threonine has been successfully marketed as a feed additive with a worldwide demand of more than 70 000 tons per year, with a price of approximately US$3 per kg.

L-Serine

The engineering of an efficient L-serine-producing bacterium is a rather recent example where a combination of cellular analyses and engineering methods resulted in a breakthrough in microbial L-serine production. L-serine is used in infusion solutions, as are all the other proteinogenic amino acids, but has no other major application. The market is therefore smaller, but the price per kg is higher than for feed additive amino acids. The current price is approximately US$40 per kg. The microbial production of L-serine originally occurred with methanol-utilizing organisms taking advantage of the serine hydroxymethyltransferase (SHMT) reaction. In the biosynthesis reaction, the SHMT catalyzes the glycine condensation with the C1-unit activated by a tetrahydrofolate derivative (THF) to form L-serine. However, this method faced a setback because of the external addition of glycine and low conversion yields. As will be seen below, the SHMT reaction plays a key role in microbial L-serine production. It catalyzes the interconversion of 5,10-methylenetetrahydrofolate + glycine + H_2O to tetrahydrofolate + L-serine, with the interconversion and dissociation of reactants within the same order of magnitude.

Using sugars like glucose as a carbon source, L-serine derives directly from glycolysis in just three enzymatic steps (**Figure 6**). A systematic study revealed that removing bottlenecks in synthesis and preventing L-serine degradation are major issues in the manufacture of L-serine. Indeed, L-serine has a key position in central metabolism since as much as 8% of the glucose carbon flux is via L-serine. The reason is that L-serine, besides being incorporated into protein, is required for a number of purposes, like synthesis of phospholipids, L-tryptophan, and L-cysteine. Moreover, the biodegradative reaction catalyzed by SHMT provides glycine required for purine, protein, and heme synthesis, and, more importantly, provides the activated C-1 compound 5,10-methylene-THF, which in turn can be further



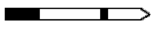
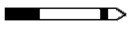
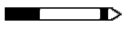
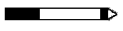
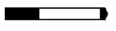
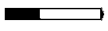
Construct	Strain	Specific activity	Residual activity (%)
	WT pZ1	0.13	38
	WT pserA	2.15	37
	WT pserAΔ79	0.19	15
	WT pserAΔ188	0.44	99
	WT pserAΔ197	1.32	92
	WT pserAΔ205	0.39	<1
	WT pserAΔ211	0.15	<1

Figure 6 On the left is shown L-serine synthesis as derived from 3-phosphoglycerate from glycolysis. On the right is shown a series of plasmid constructs and their resulting 3-phosphoglycerate dehydrogenase activity in the presence of L-serine.

converted into 5,10-methenyl-THF, and other activated C-1 units like 10-formyl-THF, 5-formyl-THF, and 5-methyl-THF to serve different demands in metabolism, like 5-methyl-THF for L-methionine synthesis, 5,10-methenyl-THF for D-pantothenate synthesis, or 10-formyl-THF for *N*-formylmethionyl-tRNA and purine synthesis. It is obvious that only because of the requirement for purine synthesis and the provision of tRNAfMet for translation initiation, a high L-serine degradative flux via the SHMT reaction occurs.

To derive from the 'workhorse' of amino acid production, *C. glutamicum*, an L-serine producer, a number of steps were undertaken. These were as follows: At first the feedback control of the 3P-glycerate dehydrogenase (PGDH) was removed, which is allosterically controlled by L-serine. Sequence comparisons revealed that the C-term of the *serA*-encoded PGDH represents the domain involved in allosteric control. Therefore, a comprehensive set of truncated *serA* versions was made, with the most prominent mutation being *serA*Δ197, where as much as 197 amino acyl residues from the C-term were deleted and which resulted in an activity almost insensitive to L-serine inhibition but with catalytic activity largely retained (**Figure 6**). However, overexpression of the mutant allele *serA*Δ197 in *C. glutamicum* either alone or in combinations with *serC* and *serB* overexpressed did not result in significant L-serine accumulation, indicating an intracellur conversion of L-serine.

Therefore, in the second step, the intracellular fate of L-serine was studied using ^{13}C-labeled L-serine. Tracing of the label revealed that L-serine as an entity is converted to pyruvate and a genomic screen revealed furthermore that an *sdaA*-encoded serine dehydratase is present in *C. glutamicum*, likely to be responsible for degradation. The serine dehydratase contains an [4Fe-4S] cluster involved in the pyridoxal-5′-phosphate-independent deamination of L-serine to pyruvate. When the enzyme was deleted and then the *serA*Δ197, *serC*, and *serB* genes overexpressed, a slight transient L-serine accumulation was observed.

The study with ^{13}C-labeled L-serine also showed that glycine was formed from the L-serine added, and this was not surprising considering that there is no preference of the SHMT for the forward or backward reaction. As mentioned, L-serine conversion catalyzed by SHMT serves to provide activated C-1 units and this cellular demand cannot entirely be bypassed by external metabolite addition. Therefore SHMT is essential. However, the metabolic engineers used a trick to deplete SHMT activity. As mentioned, SHMT requires THF for functioning. Therefore, the *pabAB* and *pabC* genes were deleted, encoding the aminodeoxychorismate synthase and aminodeoxychorismate lyase catalyzing two steps of THF synthesis. SHMT activity and growth of the resulting strain were dependent on THF, or its intermediate p-aminobenzoate.

Thus the strain eventually selected was *C. glutamicum* 13032Δ*sdaA*Δ*pabABC* p*serACB*. This is derived from the type strain ATCC13032, deleted of serine dehydratase (*sdaA*) and of the folate synthesis genes (*pabABC*), and overexpressing the three genes of the L-serine synthesis pathway (p*serACB*) with the *serA* gene carrying the deletion at its C-term. Under industrially relevant conditions the performance of this strain was demonstrated in a 20–l reactor based on corn steep liquor (CSL) medium. CSL provides some folate to enable restricted growth and reduced SHMT activity of the strain. The medium contained 35 g l^{-1} solid CSL plus initially 15 g l^{-1} glucose,

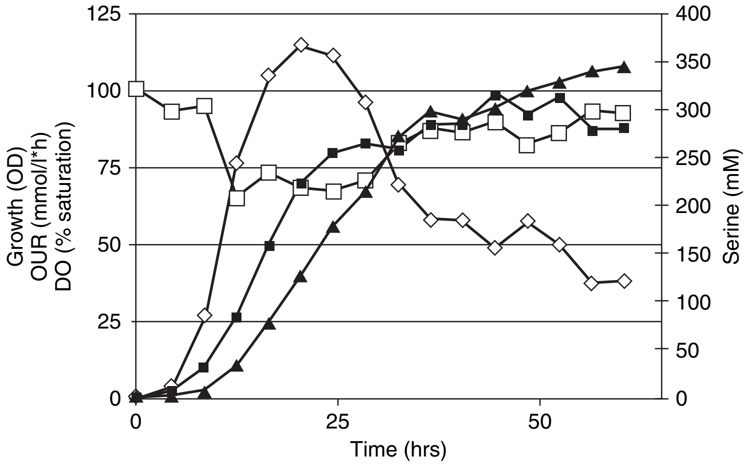


Figure 7 Performance of an L-serine producer strain in a fermenter showing growth (■), the L-serine concentration in the medium (▲), the dissolved oxygen, DO (□), and the oxygen uptake rate, OUR (◇).

15 g l^{-1} fructose, and salts. The minimum dissolved oxygen concentration was set to 50% saturation to ensure no oxygen limitation. As can be seen in **Figure 7**, inoculation of the reactor with cells enabled growth up to a maximum specific growth rate of 0.25 h^{-1}, which is about 60% that of the wild type. L-serine formation occurred from the beginning, suggesting a suitable folate supply in the culture due to CSL, which can be assumed to contain at least traces of this vitamin. The maximum oxygen uptake rate, OUR_{max}, was about 110 mol l^{-1} h^{-1}, which was present at the end of the logarithmic growth of the culture. The maximal specific productivity was 1.45 mmol g^{-1} h^{-1}, and the volumetric productivity about 1.4 g l^{-1} h^{-1}. In this experiment a final concentration of 345 mmol L-serine was reached, but significantly higher concentrations can be obtained, and further derivatives of this basic strain have been developed.

Enzymatic Production

L-Aspartic Acid

L-aspartic acid is industrially produced by an enzymatic process in which aspartase (L-aspartate ammonia lyase EC 4.3.1.1) is used. This enzyme catalyzes the reversible interconversion between L-aspartate and fumarate plus ammonia. The equilibrium constant of the deamination reaction catalyzed by the enzyme is 20 mmol l^{-1} at 39 °C and 10 mmol l^{-1} at 20 °C; thus the amination reaction is favored. Aspartase purified from *E. coli* is a tetramer with a molecular weight of 196 kDa and it has a strong requirement for divalent metal ions. As the isolated enzyme is very unstable in solution, an immobilized cell system based on *E. coli* cells entrapped in a polyacrylamide gel matrix was developed. Using this system, the half-life of the aspartase activity could be increased to 120 days. Immobilization of the cells in κ-carrageenan resulted in remarkably increased operational stability; thus, a biocatalyst with a half-life of approximately 2 years was obtained (**Table 2**). In addition, recombinant DNA techniques helped to improve aspartase-containing strains. A plasmid with the *aspA* gene elevated aspartase formation in *E. coli* approximately 30-fold.

The production of L-aspartate by means of immobilized cells has been industrialized by using a fixed-bed reactor system. A continuous process enables automation and efficient control to achieve high conversion rates and yields. A column packed with the κ-carrageenan-immobilized cells produces 200 mmol l^{-1} L-aspartate per hour per gram of cells; thus, in a 1 m^3 column about 100 tons of L-aspartate can be produced in 1 month. Compared to microbial amino acid production, the advantages of this enzymatic production method are higher product concentration and productivity. Furthermore, less byproducts are formed; thus, L-aspartic acid can be easily separated from the reaction mixture by crystallization. In recent years the market for L-aspartic acid has increased to approximately 30 000 tons per year due to the fact that

Table 2 Half-life of aspartase in *Escherichia coli* cells immobilized using various methods

Immobilization method	*Aspartase activity (U/g cells)*	*Half-life (days)*	*Relative productivity (%)*[a]
Polyacrylamide	18 850	120	100
Carrageenan	56 340	70	174
Carrageenan (GA)[b]	37 460	240	397
Carrageenan (GA + HA)[b]	49 400	680	1498

[a] Considers the initial activity, decay constant, and operation period.
[b] GA = glutaraldehyde, HA = hexamethylene diamine.

this amino acid is a precursor for the production of the dipeptide sweetener aspartame (methyl ester of aspartyl-L-phenylalanine).

Future Prospects

For the synthesis of proteinogenic amino acids, microbial fermentation plays a key role among the production methods in the amino acid industry. Because of modern techniques such as metabolic engineering combined with new analytical methods offered by the -omics techniques, like DNA chip technology, proteomics, and metabolomics, further improvements in microbial processes are constantly being achieved. Bottlenecks in L-amino acid synthesis can be removed by amplification of genes coding for the limiting enzymatic steps. The recent discovery of the L-lysine secretion carrier opens up an entirely new field for increasing the overproduction of various L-amino acids. Furthermore, a thorough understanding of the various elements and mechanisms controlling the biosynthesis of an amino acid should make it possible to further influence its production rate in a predictable way.

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Antibiotic Resistance

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Defining Statement
Introduction
Antibiotic Classes
Antibiotic Resistance
Mechanisms of Resistance
Acquisition of Resistance
Biochemistry of Resistance
Genetics of Resistance
Antibiotics Can Act as Pheromones
Biological Cost of Antibiotic Resistance
Conclusion
Further Reading

Glossary

antibiotic Molecule of microbial origin able to inhibit the growth or to kill other microorganisms.

antimicrobial agent Substance active against microorganisms but not obligatory of microbial origin. It could be synthetic, semisynthetic, or originate from plants or mammals.

chromosome DNA molecule that contains all the genetic information necessary for the life of the bacterium. Most often double-stranded, covalently closed, circular, and self-replicating.

conjugation Unidirectional transfer of genetic information (in this article, a plasmid) involving direct cellular contact between a donor (male) and a recipient (female).

integron DNA element that acquires open-reading frames embedded in gene cassette units and converts them into functional genes by ensuring their correct expression.

mutation Any inheritable alteration of DNA.

operon Adjacent genes coordinately expressed.

plasmid Minichromosome encoding accessory genetic information, such as antibiotic resistance.

replicon DNA molecule that can replicate autonomously (chromosome or plasmid)

resistance When a strain can grow in the presence of higher concentrations of the antibiotic compared to other strains of the same species.

transposon (transposable genetic element) DNA segment able to migrate from replicon to replicon (plasmid or chromosome) while retaining its physical integrity. A transposon can insert itself into nonhomologous DNA, exit, and relocate independently of the general recombination function of the host.

Abbreviations

A site	aminocyl receptor site
AAC	aminoglycoside acetyltransferases
ABC	ATP-binding cassette
ANT	aminoglycoside nucleotidyltransferases
APH	aminoglycoside phosphotransferases
CSP	competence-stimulating peptide
DHFR	dihydrofolate reductase
EF-G	elongation factor G
LPS	Lipopolysaccharide
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFS	major facilitator superfamily
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *S. aureus* strains
OM	outer membrane
Omp	OM proteins
P site	peptidyl donor site
PBPs	penicillin-binding proteins
qnr	quinolone resistance
QRDR	quinolone resistance determining region
RND	resistance-nodule-cell division
SMR	small multidrug resistance
VISA strains	vancomycin-intermediate *S. aureus* strains

Defining Statement

Resistance of bacteria to antibiotics can be intrinsic or acquired. Acquired resistance results from mutation in a gene located in the host chromosome or from horizontal acquisition of a new genetic information by conjugation or transformation. These mechanisms can be associated in the emergence and more efficient spread of resistance.

Introduction

Resistance of bacteria to antibiotics, in particular multiple resistance, has become a major health problem worldwide. Antibiotics are defined as secondary metabolites produced by microorganisms in the environment (generally the soil) active against other microorganisms because of their interaction with, and inhibition of, a specific target. In this article, the term 'antibiotic' is used to designate natural, but also semisynthetic (e.g., certain aminoglycosides) or entirely synthetic (e.g., quinolones), molecules with antibacterial activity.

Antibiotic Classes

Antibiotics are grouped in classes (or families) on the basis of their chemical structure, for example, ß-lactams (penicillins and cephalosporins), aminoglycosides (streptomycin, kanamycin, and gentamicin), and tetracyclines. As a consequence, members of a given class are closely related and generally share the same target in the cell and are thus substrates for the same mechanism of resistance. As will be discussed below, this implies that the reasoning in terms of resistance should be in classes rather than in individualized antibiotics.

The main classes of antibiotics act on four different targets (**Table 1**).

Inhibition of Cell Wall Biosynthesis

The cell wall protects prokaryotes from the environment and from osmolysis. The bacterial cell wall is characterized by the presence of a peptidoglycan located outside the cytoplasmic membrane that is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. Synthesis of the cell wall requires several steps in the cytoplasm, such as synthesis of the muramyl pentapeptide, which is then translocated to the external face of the cell membrane by a carrier. Cross-linking by transglycosylases and transpeptidases is responsible for the complete synthesis of the peptidoglycan outside of the cell.

The β-lactam antibiotics, such as penicillins and cephalosporins, block the transpeptidation events by binding to the transpeptidases, also named penicillin-binding proteins (PBPs).

Glycopeptides act by binding, in a noncovalent fashion, to the C-terminal of D-alanine-D-alanine dipeptide of the peptidoglycan precursors, preventing their incorporation into the growing wall.

Fosfomycin inhibits the activity of MurA, an enzyme implicated in the conversion of the nucleotide diphosphosugar UDP-*N*-acetylglucosamine into UDP-muramyl pentapeptide, by binding to its active site cysteine and thus blocking the formation of muramyl pentapeptide.

Inhibition of Protein Biosynthesis

Bacterial ribosomes, which translate the mRNA in amino acid sequences, are constituted of two subunit nucleoprotein particles, named 50S and 30S. The large 50S subunit contains proteins and two rRNA, 23S and 5S, whereas the small 30S subunit is composed of proteins and the 16S rRNA. The translation events start with the binding of mRNA to the 30S subunit. A formylmethionyl-tRNA is then attached to the peptidyl (P) donor site face of the AUG initiator codon. The 50S subunit is then added, and the adequate aminoacyl-tRNA enters the aminocyl receptor (A) site, which is adjacent to the peptidyl donor site (P site). A specific peptidyl transferase mediates a peptide bond between the *N*-formylmethionine and the adjacent amino acid.

Macrolides, such as erythromycin, bind to the 23S rRNA, near the peptidyl transferase center, block the

Table 1 Targets of the main classes of antimicrobial drugs

Cell wall synthesis	*Protein synthesis*	*DNA replication*	*Membrane*
Bacitracin	Aminoglycosides	Coumarines	Polymixins
β-Lactams	Chloramphenicol	Quinolones	
Glycopeptides	Fusidic acid	Rifampin	
Fosfomycin	Ketolides	Sulfonamides	
	Lincosamides	Trimethoprim	
	Macrolides		
	Oxazolidinones		
	Streptogramins		
	Tetracyclines		

entrance of the ribosomal tunnel, and thus stop the elongation of the peptide chain.

Tetracyclines bind in the vicinity of the A site of the 30S subunit and block the moving of the tRNA along the ribosome, which impedes the formation of the first peptide bond.

Chloramphenicol binds to the A site and prevents binding by tRNA.

Lincosamides (lincomycin and clindamycin), by interacting with both the A site and the P site inhibit peptide bond formation.

The ribosome is also the specific target of aminoglycosides that act by causing translational errors and by inhibiting translocation.

Inhibition of DNA Replication

Topoisomerases are essential for cell viability. DNA gyrase is implicated in the control of DNA topology, in DNA replication, recombination, and transcription. Topoisomerase IV is involved in DNA replication and decatenation of the chromosome. Interaction of quinolones with enzyme-bound DNA complexes is responsible for conformational changes and accumulation of complexes that could block the replication fork.

Rifampin is an RNA polymerase inhibitor that hinders protein transcription of DNA into mRNA.

Other Targets

Folic acid is an essential precursor in nucleic acid synthesis. Trimethoprim–sulfamethoxazole inhibits the folic acid metabolism pathway: the first molecule blocks the dihydrofolate reductase (DHFR), an essential enzyme for DNA synthesis, whereas the second blocks the dihydropteroate synthase.

Polymixins increase the permeability of the cell membrane.

Antibiotic Resistance

There are two major types of resistance to antibiotics: intrinsic and acquired.

Intrinsic Resistance

Intrinsic (or natural) resistance is present in all the bacteria of a given species or genus and could thus be better considered as insensitivity. It delineates the spectrum of activity of an antibiotic. This type of resistance could be the result of the physiological characteristics of the bacterial species or of the presence of a structural gene. Natural resistance is often due to (1) inaccessibility of the target by antibiotics, (2) low affinity of the antibiotics for the target, or (3) absence of the target. For example, presence of an external membrane in Gram-negative bacilli (such as *Escherichia coli*) leads to resistance to various drug classes (glycopeptides, macrolides, lincosamides, streptogramins, etc.) due to impermeability.

Pseudomonas aeruginosa is a typical organism that exhibits a high broad substrate range intrinsic resistance resulting from a particularly low permeability of its outer membrane (OM) associated with a number of endogeneous multidrug efflux systems (such as MexAB-OprM and MexXY-OprM) and a chromosomally encoded β-lactamase (AmpC).

Enterococcus faecium produces an intrinsic low affinity PBP 5 responsible for high level resistance to cephalosporins, oxacillin, and monobactams and for an increase in resistance to the penicillins and carbapenems. *Enterococcus* spp. are also intrinsically resistant to low levels of aminoglycosides, due to inefficient uptake of this class of antibiotics. As already mentioned, glycopeptides, vancomycin and teicoplanin, inhibit cell wall synthesis in Gram-positive bacteria by binding to the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residues of late pentapeptide peptidoglycan precursors. *Enterococcus gallinarum* and *Enterococcus casseliflavus* and *Enterococcus flavescens* are intrinsically resistant to low levels of glycopeptides by synthesis of modified peptidoglycan precursors ending in D-alanine-D-serine (D-Ala-D-Ser), for which glycopeptides have a low affinity, and by elimination of the D-Ala-D-Ala ending precursors. These two concomitant events are due to a chromosomally encoded *vanC* gene cluster.

Resistance to both β-lactams and cyclines in Mycobacteria is due to the combination of reduced permeability of the bacterial cell wall, presence of modifying enzymes, and low affinity for the target (such as PBP and DNA gyrase).

Acquired Antibiotic Resistance

Acquired resistance is present only in some strains of the same species or genus. In certain instances, it can be highly prevalent, such as penicillinase production in staphylococci is present in more than 90% of the strains. Intrinsic and acquired resistances do not differ in their mechanisms; both can employ the four major pathways depicted in **Figure 1**.

Mechanisms of Resistance

On a biochemical point of view, bacteria have developed four major mechanisms of resistance (**Figure 1**): (1) modification of the target, which leads to loss or decrease in affinity of the drug for its target or synthesis of a new target; (2) production of an enzyme that will inactivate or modify the drug; (3) impermeability, in particular by loss of a porin (pore in the external membrane) or by

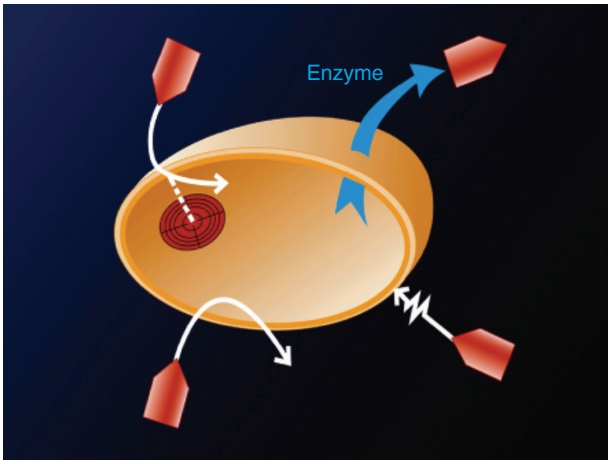

Figure 1 Major mechanisms of resistance to antibiotics. From top, counterclockwise, alteration of the target; production of an enzyme inactivating the drug; impermeability by mutation in a porin channel; impermeability by active efflux of the drug.

diminution of its diameter in Gram-negative bacteria; and (4) efflux of antibiotics outside of the cells by energy-dependent pumps. The common motif of these various mechanisms is to impede interaction of the antibiotic with its target.

Alteration or Synthesis of a New Target

A mechanism frequently used by bacteria to prevent the action of antimicrobial agents is the alteration of specific targets that have a necessary role in microbial growth. Enzymes involved in several steps of peptidoglycan synthesis, such as PBPs, or assembly represent targets of choice for antibiotics. Alteration of PBPs, resulting in low affinity for β-lactams, or acquisition of new PBPs is responsible for β-lactam resistance. As an example, resistance to this antibiotic class in *Streptococcus pneumoniae* is mainly due to alteration of PBPs. *S. pneumoniae* has six PBPs (1a, 1b, 2a, 2b, 2x, and 3) in which point mutations could be responsible for β-lactam resistance in mutants obtained *in vitro*. In clinical isolates, resistance is due to low-affinity variants of PBPs 2b, 2x, and 1a that are encoded by mosaic genes that result from DNA acquisition by transformation from related species of *streptococci* that are intrinsically less susceptible to β-lactams followed by homologous recombination. Genesis of these mosaic PBP genes is facilitated by the fact that *S. pneumoniae* is naturally transformable. Competence, which is the ability to take up DNA from the environment, in *S. pneumoniae* is due to a specific protein, the competence-stimulating peptide (CSP), which acts as a pheromone. In response to a certain population density, the production of CSP is increased by the upregulation of the *comC* or *comA* gene. A typical example of a mosaic PBP-mediated resistance is the mosaic PBP 2x, which is the result of the replacement of a portion of PBP 2x of *S. pneumoniae* by the corresponding portion of the gene from *Streptococcus oralis* or *Streptococcus mitis*. This mosaic PBP 2x is more resistant to cefotaxime, despite the fact that the donor strain and the recipient *S. pneumoniae* were cefotaxime susceptible. Other mosaic structures responsible for synthesis of low affinity PBP variants have been described (PBP 1a, 1b, 2a, and 2b). Alteration of three or more PBPs is found in highly resistant isolates.

High level resistance to methicillin and all other β-lactams in *Staphylococcus aureus* (methicillin-resistant *S. aureus* strains (MRSA)) is due to acquisition of a gene, *mecA*, responsible for synthesis of a new PBP, PBP 2a, with reduced affinity for β-lactams. In the absence of *mecA*, low level resistance could be due to overproduction of PBP 4 or due to modification of PBP 2.

Acquired resistance to glycopeptides in *E. faecium* and *Enterococcus faecalis* detected in 1986 is due to acquisition of operons encode enzymes producing modified peptidoglycan precursors terminating in D-Ala-D-lactate (D-Ala-D-Lac) (VanA, VanB, and VanD-type resistance) or in D-Ala-D-Ser (VanE, and VanG-type). Interestingly, the base composition mol% GC of the genes in the *van* operons suggests that these clusters are composed of genes from various sources (**Figure 2**). The first clinical isolates of MRSA that have acquired a *vanA* gene cluster were detected in 2002. Vancomycin resistance in vancomycin-intermediate *S. aureus* (VISA strains) is not due to acquisition of a *van* gene cluster but due to synthesis of a thicker cell wall that traps vancomycin, leading to a reduced number of molecules that reach the transglycosylase targets located in the cytoplasmic membrane.

The macrolide, lincosamide, and streptogramin B antibiotics act by binding to the 50S ribosomal subunit and thus prevent protein synthesis. Resistance to these molecules is due to the methylation of an adenine residue (A2058 in *E. coli*) in 23S rRNA by a methyltransferase specified by an *erm* (erythromycin ribosome methylation) gene. Addition of a methyl group reduces the affinity of the rRNA for these three groups of antibiotics that have very different structures. Enzymatic methylation of 16S rRNA by other methylases of the Rmt family (RmtA, B, C, and D) or ArmA confers high level resistance to aminoglycosides.

Alteration of the targets of fluoroquinolones type II topoisomerases, DNA gyrase and topoisomerase IV, constitutes the main mechanism of resistance to these antimicrobial agents. These two enzymes, implicated in bacterial DNA synthesis, are composed of two subunits (GyrA and GyrB for DNA gyrase and ParC and ParE for topoisomerase IV). Mutations in a specific region of these subunits, the QRDR (quinolone resistance determining region), prevent the fixation of the quinolones on the DNA–enzyme complex. The levels of resistance conferred by mutations in the subunits of DNA gyrase or

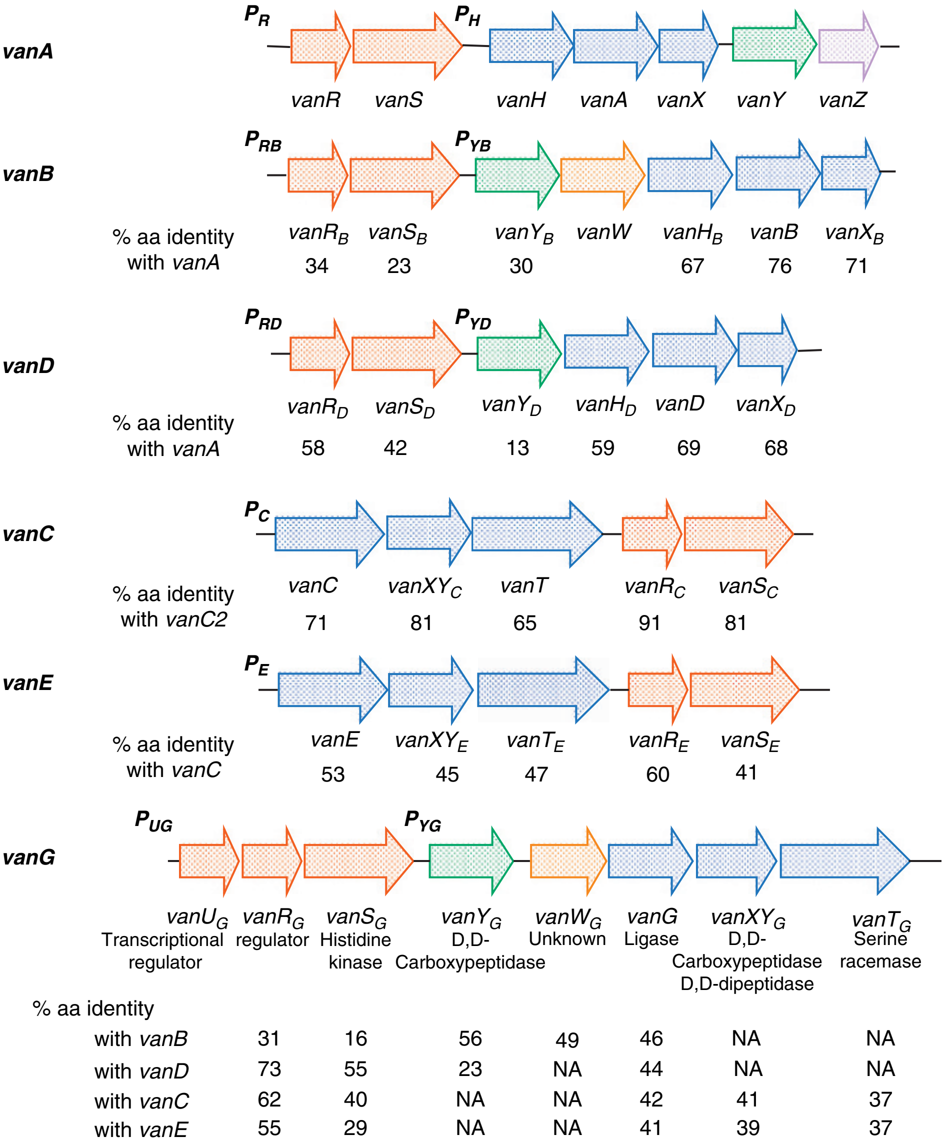
vanA
P_R
P_H
vanR vanS vanH vanA vanX vanY vanZ
vanB
P_{RB}
P_{YB}
% aa identity with vanA
$vanR_B$ 34
$vanS_B$ 23
$vanY_B$ 30
vanW
$vanH_B$ 67
vanB 76
$vanX_B$ 71
vanD
P_{RD}
P_{YD}
% aa identity with vanA
$vanR_D$ 58
$vanS_D$ 42
$vanY_D$ 13
$vanH_D$ 59
vanD 69
$vanX_D$ 68
vanC
P_C
% aa identity with vanC2
vanC 71
$vanXY_C$ 81
vanT 65
$vanR_C$ 91
$vanS_C$ 81
vanE
P_E
% aa identity with vanC
vanE 53
$vanXY_E$ 45
$vanT_E$ 47
$vanR_E$ 60
$vanS_E$ 41
vanG
P_{UG}
P_{YG}
$vanU_G$ Transcriptional regulator
$vanR_G$ regulator
$vanS_G$ Histidine kinase
$vanY_G$ D,D-Carboxypeptidase
$vanW_G$ Unknown
vanG Ligase
$vanXY_G$ D,D-Carboxypeptidase D,D-dipeptidase
$vanT_G$ Serine racemase
% aa identity
with vanB 31 16 56 49 46 NA NA
with vanD 73 55 23 NA 44 NA NA
with vanC 62 40 NA NA 42 41 37
with vanE 55 29 NA NA 41 39 37

Figure 2 Comparison of the *van* gene cluster. Open arrows represent coding sequences and indicate the direction of transcription. NA, not applicable.

topoisomerase IV depend on both the bacterial species and the quinolone. The primary target is the DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. Mutations in the GyrA or ParC subunits are more common than mutations in GyrB or ParE. Another resistance mechanism to fluoroquinolones is mediated by the plasmid-borne *qnr* (quinolone resistance) gene. The Qnr gene product, which belongs to the pentapeptide repeat family, protects gyrase and topoisomerase IV from quinolone inhibition by binding these enzymes directly. To date, three types of *qnr* genes have been described, *qnrA*, *qnrB*, and *qnrS*. Within each type, several variants have been reported.

Antibiotics of the rifamycin family, such as rifampin, interact with the β subunit of RNA polymerase, which is encoded by the *rpoB* gene, and block transcription initiation. Mutations, including point mutations, insertions, and deletions, responsible for rifampin resistance are located in highly conserved regions, notably between the residues 507 and 534. Mutations in *rpoB* have been detected in Mycobacteria, *S. pneumoniae*, *S. aureus*, and *Neisseria meningitidis*.

Mutations in the *dhfr* gene, encoding a DHFR, are responsible for trimethoprim resistance in staphylococci.

Alteration of other targets, such as iso-leucyl-tRNA synthetase, elongation factor G (EF-G), and NADH-enoyl-ACP-reductase/β-ketoacyl-ACP-synthase, is responsible for resistance to mupirocin, fusidic acid, and isoniazid, respectively.

Enzymatic Modification

This is a major mechanism of resistance to antibiotics such as β-lactams, macrolides, aminoglycosides, and chloramphenicol.

β-Lactamases hydrolyze the four-membered β-lactam ring in penicillins, cephalosporins, carbapenems, and monobactams. The enzymes can be classified in a number of ways, such as by their amino acid sequences or by their enzymatic activity spectrum. In the latter classification, four groups have been defined: group 1, cephalosporinases on which the β-lactamase inhibitor clavulanic acid has a weak activity; group 2, penicillinases sensitive to clavulanic acid and extended spectrum β-lactamases; group 3, metallo-β-lactamases; and group 4, other β-lactamases weakly sensitive to clavulanic acid.

The macrolide esterases, such as EreA and EreB, inactivate macrolides by cleaving the macrocycle ester.

The transferase group (phospho-, nucleotidyl-, acetyl-, thiol-, ADP-ribosyl-, and glycosyltransferases) constitutes a large family of modifying enzymes. The phosphotransferases catalyze the transfer of a phosphate group (generally from ATP) to a substrate. The aminoglycoside phosphotransferases (APH) confer a higher level of resistance than aminoglycoside acetyltransferases (AAC) or aminoglycoside nucleotidyltransferases (ANT). Depending on the aminoglycoside modification, more than 50 antibiotic-inactivating enzymes have been reported. Furthermore, various aminoglycoside-inactivating enzymes can be present in the same host. A remarkable example of this coexistence is the bifunctional enzyme AAC(6′)-APH(2″), which is the fusion product of two genes and possesses an acetyl and a kinase activity in the same protein. This enzyme is responsible for high level resistance in Gram-positive bacteria to all the aminoglycosides, except streptomycin and spectinomycin. Resistance to chloramphenicol is due to a large variety of chloramphenicol acetyltransferases, which are widely distributed among bacterial pathogens of all genera. Fosfomycin is modified by thiol transferases such as FosA, FosB, or FosX. FosA, found in Gram-negative bacteria, is present on the chromosome of *P. aeruginosa* whereas FosB is found in Gram-positive bacteria and, notably, on the chromosome of *Bacillus subtilis*. Three genes, *mph(A)*, *mph(B)*, and *mph(D)*, encoding macrolide 2′-phosphotransferases have been reported in *E. coli* and *Pseudomonas*.

Reduced Uptake of Antibiotics

Gram-negative bacteria possess an OM external to the peptidoglycan and composed of lipopolysaccharide (LPS) and phospholipids. This OM functions as an effective barrier, the LPS being responsible for impermeability of the OM to many molecules. Thus, some OM proteins (Omp), also called porins and acting as aqueous channels, are used by several antibiotics, such as β-lactams, chloramphenicol, or fluoroquinolones, to permeate the OM. Resistance to these families of antibiotics could be due to the diminution of the porin copy number or reduction in the size of the pore.

The OM of *P. aeruginosa* has a very low permeability to small hydrophilic molecules, allowing resistance of this organism to fluoroquinolones. Resistance to imipenem in *P. aeruginosa* is caused by a loss of the OprD porin in response to exposure to this antibiotic. Other antibiotic resistances associated with loss of a porin have been documented in *Serratia marcescens*, *E. coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella pneumoniae*.

Increased Efflux of Antibiotics

The widespread active export or efflux of antibiotics outside bacteria limits intracellular accumulation of toxic compounds such as antibiotics. This mechanism is mediated of membrane-based efflux proteins acting as pumps. Efflux pumps have a narrow (such as tetracycline pumps) or a broad specificity, the latter conferring a multidrug resistance (MDR) phenotype to chemically and structurally unrelated compounds. Generally, drug-specific efflux pumps are encoded by mobile genetic elements, whereas MDR efflux pumps are specified by the chromosome. To date, five families of efflux systems have been described: the major facilitator superfamily (MFS), the ATP-binding cassette (ABC), the resistance-nodule-cell division (RND), the small multidrug resistance (SMR), and the multidrug and toxic compound extrusion (MATE) (**Table 2**).

Drug efflux systems act in an energy-dependent manner by using ATP hydrolysis (ABC) or an ion antiport mechanism (MFS, RND, SMR, and MATE). The expression of multidrug transporters is commonly controlled by specific regulatory proteins. Antibiotic resistance in an efflux mutant is due to overexpression of an endogeneous pump or due to a mutation in a protein pump that enhance the potential of export of this protein.

As opposed to ABC transporter that usually mediates the export of specific antimicrobial classes, the MFS, RND, SMR, and MATE pumps, also designed as secondary drug transporters, are generally responsible for resistance to multiple antimicrobial agents.

Efflux pump specific for one substrate

Tetracyclines inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracycline-specific efflux pumps, which are members of the MFS family, are found in both pathogenic Gram-negative and Gram-positive bacteria. The *tet* efflux genes code for membrane-associated proteins that reduce the intracellular drug concentration and thus protect the

Table 2 Typical substrates of the five classes of antibiotic efflux pumps

MFS	*RND*	*SMR*	*MATE*	*ABC*
Aminoglycosides	Aminoglycosides	Aminoglycosides	Aminoglycosides	Aminoglycosides
Chloramphenicol	β-lactams	Chloramphenicol	Fluoroquinolones	Chloramphenicol
Erythromycin	Chloramphenicol	Erythromycin		β-lactams
Fluoroquinolones	Erythromycin	Tetracyclines		Erythromycin
Lincosamides	Fluoroquinolones			Fluoroquinolones
Novobiocin	Novobiocin			Lincosamides
Rifampin	Rifampin			Macrolides
Tetracyclines	Tetracyclines			Novobiocin
	Trimethoprim			Tetracyclines

ABC, ATP-binding cassette; MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; RND, resistance-nodule-cell division; SMR, small multidrug resistance.

ribosome. Most of the *tet* determinants are found on mobile elements.

Macrolides also inhibit bacterial growth by binding to ribosomes. Efflux is also implicated in macrolide resistance. The *mef* genes, initially described in *S. pneumoniae* and *Streptococcus pyogenes*, are implicated in the specific efflux of 14- and 15-membered macrolides. The *msr(A)*/*msr(B)* and *msr(C)* genes, detected in *Staphylococcus* spp. and *E. faecium*, respectively, are responsible for macrolide/streptogramin efflux.

The *cmlA* genes, which are also widespread among Gram-negative bacteria, encode exporters of the MFS family that confer chloramphenicol resistance.

Efflux pump associated with MDR

Major facilitator superfamily

Overproduction of NorA, a chromosomally encoded protein of the MFS family, is responsible for quinolone resistance in *S. aureus*. NorA is homologous to Bmr of *B. subtilis* and PmrA of *S. pneumoniae*, the latter being responsible for an increase of the norfloxacin minimal inhibitory concentration (MIC). A fluoroquinolone efflux gene, named *qepA* and located on a plasmid detected in *E. coli*, specifies an MFS transporter that confers low level of resistance to the hydrophilic quinolones norfloxacin and ciprofloxacin.

An efflux pump, Tap, conferring resistance to aminoglycosides and the tetracyclines, has been detected in Mycobacteria, such as *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*.

The chromosomally-encoded efflux pump EmrAB confers in *E. coli* resistance to nalidixic acid and to other toxic compounds. A homologous pump, VceAB, was found in *Vibrio cholerae*, a Gram-negative enteric pathogen.

An endogenous gene of *Listeria monocytogenes*, *lde*, encodes a protein of the MFS family and is responsible for fluoroquinolone resistance.

Resistance-nodule-cell division

The RND family constitutes the most important multidrug efflux systems for clinically important antimicrobials. This family can accommodate a broad range of structurally unrelated molecules.

In addition to intrinsic resistance by impermeability, *P. aeruginosa* also expresses efflux systems of the RND family. As already mentioned, some of them (such as MexAB-OprM or MexXY-OprM) participate in the intrinsic resistance. MexAB-OprM contributes to resistance to quinolones, chloramphenicol, β-lactams, novobiocin, trimethoprim, macrolides, tetracycline, and the biocide triclosan. The MexCD-OprJ system is implicated in fluoroquinolone resistance but also accommodates β-lactams, chloramphenicol, macrolides, tetracycline, and trimethoprim.

In *E. coli*, the AcrAB-TolC system is homologous to MexAB-OprM. Substrates of AcrAB-TolC include macrolides, chloramphenicol, fluoroquinolones, tetracycline, rifampin, lipophilic β-lactams, fusidic acid, ethidium bromide, and triclosan. AcrD and AcrEF also encode efflux pumps. AcrA, B, D, and F are also present in the *Salmonella enterica* genome.

In *Acinetobacter baumannii*, overexpression of AdeABC, an RND tripartite efflux pump, is responsible for resistance to aminoglycosides and decreased susceptibility to chloramphenicol, fluoroquinolones, erythromycin, tetracycline, trimethoprim, meropenem, and the dye ethidium bromide. Expression of the *adeABC* operon is regulated by a two-component regulatory system, *adeRS*. Mutations in *adeR* or *adeS* are responsible for the constitutive expression of the AdeABC pump, which is otherwise cryptic in wild-type *A. baumannii*.

Multidrug and toxic compound extrusion

Two homologous pumps, NorM (*Vibrio parahaemolyticus*) and YdhE (*E. coli*), are implicated in fluoroquinolone and aminoglycoside resistance.

Small multidrug resistance

Substrates of these SMR pumps, detected in *S. aureus* (Smr) and *E. coli* (EmrE), include disinfectants and antiseptics. Genes coding for QacE and QacEΔ1, responsible for quaternary ammonium compounds resistance, are found in Gram-negative and in both Gram-negative and Gram-positive bacteria, respectively, and are located in integrons.

ATP-binding cassette

Most bacterial ABC drug transporters are implicated in the export of specific antibiotics and have been described in various species. Expression of *lmrA* from *Lactococcus lactis* in *E. coli* is responsible for resistance to aminoglycosides, lincosamides, macrolides, quinolones, streptogramins, tetracyclines, and chloramphenicol. Similarly, the *E. faecalis efrAB* gene is responsible for norfloxacin and ciprofloxacin resistance; expression of *vcaM* of *V. cholerae* renders bacteria resistant to tetracycline, norfloxacin, and ciprofloxacin.

Acquisition of Resistance

On a genetic point of view, resistance can be acquired by two totally distinct events: Either occurrence of a mutation in the genome leading to vertical inheritance of resistance to the progeny of the bacterium or acquisition of foreign genetic information, from other bacteria, by horizontal transfer.

There is a multiplicity of definitions of resistance and we have already considered the intrinsic and acquired types. Genetic resistance is when the daughter cell differs from the parental cell by a genetic event (following a mutation or horizontal acquisition of genetic information). Biochemical, in bacteria that differ by the presence or the absence of a resistance mechanism. Microbiologic, when a strain can tolerate a significantly higher concentration of antibiotic (generally expressed as MIC in $\mathrm{mg\,l^{-1}}$). Clinical, which is based on the clinical outcome: success or failure of antibiotic therapy in a patient suffering from a bacterial infection. For the sake of clarity, only the genetic dimension of resistance is considered; in other words, the resistant bacterium (daughter cell) has suffered a genetic alteration relative to its parent (mother cell).

Biochemistry of Resistance

Cross-Resistance

Cross-resistance corresponds to resistance to all the antibiotics belonging to the same class due to a single mechanism. As we have seen above, drugs assigned to a same class are chemically related, have thus the same target of action in the cell, and are therefore subject to cross-resistance: bacteria that are resistant to one member of the class are generally resistant to the other members. However, there are degrees in cross-resistance: the more active the drug, the lower the level of resistance. In general, drugs recently developed are more active than old molecules of the same class. For example, among quinolones, ciprofloxacin is much more active than nalidixic acid. As a result, Gram-negative bacteria that have suffered a mutational event in the target of quinolones, the type II topoisomerases (DNA gyrase and topoisomerase IV) become much more resistant to nalidixic acid (that has high MICs) than to ciprofloxacin (that retains lower MICs). This observation stresses that a resistance mechanism has no absolute value. The level of resistance also depends on the degree of susceptibility of the host bacterium. Resistance by a given mechanism will be much higher if the bacterial species is poorly susceptible. For example, the same mechanism will confer to *P. aeruginosa*, a species naturally poorly susceptible to antibiotics, which has a resistance level much higher than *N. meningitidis*, a species exquisitely susceptible to drugs. Importantly, cross-resistance implies cross-selection: use of a given antibiotic can select resistance to other members of the class but not to drugs belonging to other classes.

Co-resistance

In co-resistance, various mechanisms are associated in the same bacterial host, sometimes stabilized by integration into the genome. Each confers (by cross-resistance) resistance to a class of antibiotics which, in fine, can result in a broad spectrum of resistance (MDR). Again, the consequence of co-resistance is co-selection. Use of a member of a drug class can co-select resistance to another class of antibiotics with a totally distinct mode of action. This is, for example, the case for *S. pneumoniae* (**Table 3**).

In France in 1999, among clinical isolates of pneumococci, 46% of the strains were susceptible to penicillin G whereas 54% were resistant to this antibiotic. If one compares the rates of resistance of these two groups of bacteria to other drug classes (first two rows, **Table 3**), it is apparent that the strains resistant to penicillin G are much more often resistant to the other classes of antibiotics. If now one considers exclusively the penicillin G-resistant strains (considered as 100%, column 2 in **Table 3**), one realizes that resistance to other classes of antibiotics is extremely high. For example, administration of the trimethroprim–sulfamethoxazole combination (Bactrim, the antibiotic most prescribed worldwide) has 88% chances to co-select a pneumococcus strain resistant to penicillin G, although these two drugs have totally different structures and targets of action.

Integrons are the most efficient way to achieve co-resistance (**Figure 3**). These elements represent a very elegant genetic system for capture and expression of resistance genes. Integrons, which can be located either in the chromosome or in plasmids, are composed of genes that have been acquired by site-specific recombination. They possess the machinery necessary to capture exogeneous genes: an integrase (*intI*) that allows recombination of circularized DNA (gene cassettes), a recombination site (*attI*),

Table 3 Antibiotic resistance in *Streptococcus pneumoniae*

	Resistant to (%)				
S. pneumoniae (%)	***PenG***	***Em***	***Cm***	***Tc***	***Tp-Su***
Pen^{S} (46%)	0	20	14	15	10
Pen^{R} (54%)	100	80	38	51	66
Em^{R}	82	100			
Cm^{R}	77		100		
Tc^{R}	80			100	
Tp-Su^{R}	88				100

Cm, chloramphenicol; Em, erythromycin; PenG, penicillin G; R, resistant; S, susceptible; Su, sulfonamides; Tc, tetracycline; Tp–Su, trimethoprim–sulfamethoxazol (Bactrim).

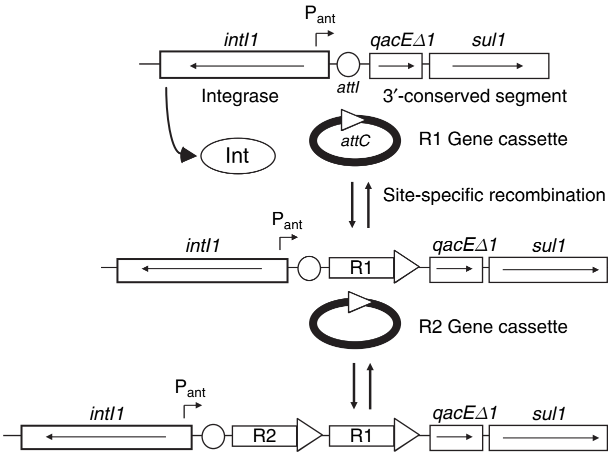

Figure 3 Model for site-specific gene cassette integration-excision in an integron. *attI*, attachment site of the integron; *attC*, attachment site of the cassette; *intI1*, gene for the integrase; Int, integrase; P_{ant}, outward-oriented promoter for the cassettes. Horizontal arrows indicate sense of transcription.

and a promoter (P_{ant}) that directs transcription of the captured genes. The resistance gene cassettes inserted into the integron contain a single gene and, downstream from it, a specific *attC* site, which is an imperfect inverted repeat. In presence of the integron-encoded integrase, a gene cassette containing *attC* inserts by site-specific recombination at the *attI* site and the gene is transcribed from the P_{ant} promoter. After integration of a gene cassette, another one can be inserted at the *attI* site. This integrative process is reversible. There is a clear relationship between the position of a cassette in the integron and the level of resistance: the closer of P_{ant}, the higher level of expression of the resistance gene. To date, five classes of integrons implicated in the dissemination of antibiotic resistance genes have been reported. Class 1 integrons, in which most of the antibiotic resistance gene cassettes can be found, have been detected in many Gram-negative and, less frequently, in Gram-positive bacteria. Gene cassettes in class 1 integrons confer resistance to β-lactams, aminoglycosides, erythromycin, chloramphenicol, trimethoprim, fosfomycin, lincomycin, and antiseptics of the quaternary ammonium compound family. The resistance determinants are tightly linked, because they are not only adjacent, but co-expressed from the same promoter. Since the genetic organization of integrons results in co-expression of the genes that have been integrated, use of any antibiotic that is substrate for one of the resistance mechanisms will co-select for resistance to all the others. The emergence of new gene cassettes in class 1 integrons, such as *qnr* implicated in resistance to fluoroquinolones, is of concern.

Extended Cross-Resistance

This type of resistance is due to a single mechanism (therefore cross-resistance is dealt) that can confer resistance to various drug classes and is thus designated as 'extended' cross-resistance. As in co-resistance, but with differences in genetic and biochemical organization, a class of antibiotics can select for resistance to other drug classes. A typical example is the methylation of a specific adenine residue in 50S rRNA that confers high level resistance to macrolides, lincosamides, and streptogramins B although these three classes have different chemical structure.

Another example of this type of resistance is overexpression of efflux pumps that can have very broad substrate ranges. The pumps that are grouped in superfamilies use energy provided by the protonmotive force or hydrolysis of ATP. In Gram-negative bacteria, the RND pumps can export a large array of antibiotic molecules, with very different structures, and also biocides such as triclosan. This accounts for the fact that detergents, that are increasingly used in household products, can select multiresistant bacteria. The pumps should be considered as the kidneys of the bacteria since they export molecules that are toxic, in particular, products of the cellular catabolism. The chromosomal structural genes for the pumps are positively or negatively regulated and are generally expressed at low level. A mutation in one of the genes involved in regulation (activator, repressor, or two-component regulatory system) or in the operator will result in overexpression of the pump and leads to resistance to its various substrates. Thus, the smallest genetic event, a point mutation, can lead in one step to resistance to a large set of antibiotics, the so-called MDR.

Genetics of Resistance

The genome of bacteria is constituted of the chromosome and accessory mobile genetic elements such as plasmids and transposons (**Figure 4**). The chromosome contains all the genetic information required for the life cycle of the bacteria. In general, the chromosome is not self-transferable horizontally to other bacteria. Chromosomal resistance genes and mutated genes involved in drug resistance are inherited vertically by the next generation of bacteria. Plasmids and transposons encode functions that are not strictly required for bacterial life but that can provide advantages to the host. Antibiotic resistance genes are only transiently useful to bacteria and it thus makes sense that they are often transferable and part of mobile genetic elements. In fact, any gene can be part of a volatile structure as long as it provides intermittent selective advantage to the host and that adequate selective pressure exerts. These mobile genetic elements can be inherited horizontally and vertically.

Plasmids

Plasmids are extrachromosomal elements that can be transferred laterally by conjugation or by mobilization. They may carry resistance to several antibiotics. Conjugative plasmids are self-transferable from cell to cell by conjugation, a mechanism that requires physical contact between the donor and the recipient bacterium (**Figure 5**). Mobilizable plasmids can be transferred with the help of a conjugative plasmid coresident in the same cell. A plasmid can carry multiple, easily up to seven, resistance determinants. Because of this physical linkage, selection for resistance to any of them will lead to co-transfer of the others. Thus, in a single genetic event, conjugation, a bacterium can acquire 'en bloc' a multiplicity of resistances.

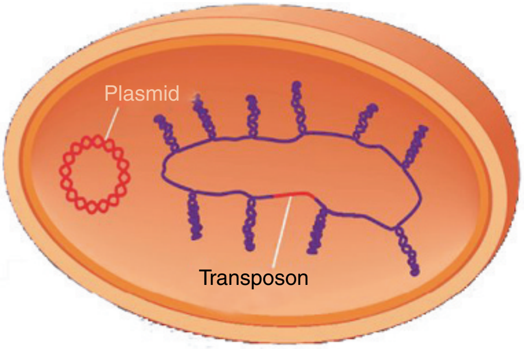

Figure 4 Schematic representation of the bacterial genome.

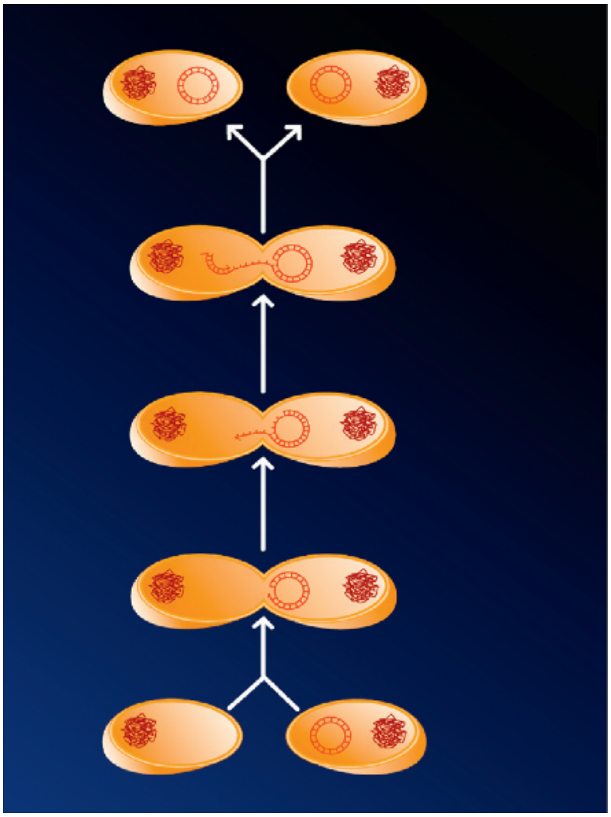

Figure 5 Schematic representation of plasmid conjugation. Bottom right, donor bacterium; bottom left, recipient bacterium. The chromosomes are represented in a condensed state. After a single nick on one of the two complementary DNA strands of the plasmid, one strand is transferred from the donor to the recipient. During this process, the complementary strand of the remaining DNA strand in the donor is synthesized while the complementary strand of the incoming DNA is synthesized in the recipient. After transfer, each bacterium contains a copy of the plasmid (top) and can therefore act, in turn, as a donor.

Transposons

Transposons are DNA fragments that can migrate from one replicon to another while retaining their structural integrity (**Figure 6**). Transposons encode a transposase that allows site-specific insertion and excision. They can transfer actively, in the case of conjugative transposons of Gram-positive cocci, or passively when they are borne by a transferable plasmid. Integrons, as described above, are found frequently as part of transposons, and transposons are frequently carried on plasmids. Numerous plasmids and transposons carry antibiotic resistance genes, often several of them. Furthermore, mobile genetic elements carrying antibiotic resistance genes often encode resistance to heavy metals and detergents. Thus, selection pressure exerted by biocides may select for antibiotic resistance.

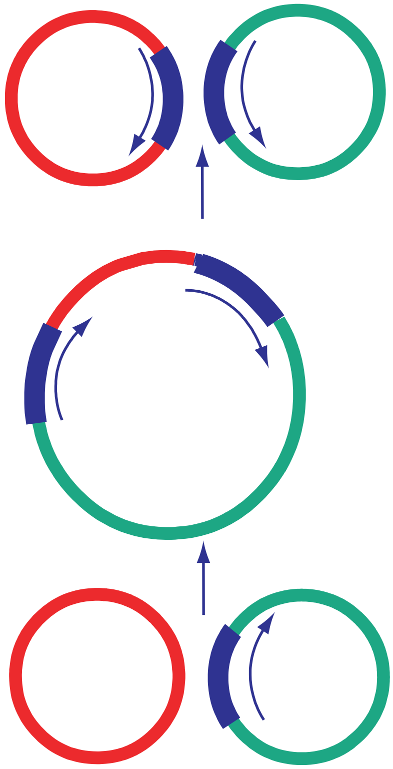

Figure 6 Replicative transposition. The donor replicon (lower right) contains a copy of the transposon (close bar; the direction of replication is indicated by an arrow) whereas the acceptor replicon (lower left) does not. Selective replication of the transposon and replicon fusion generate a bireplicon that contains two copies of the element, in the same orientation, at the borders of the replicons (middle). Following recombination between the two copies of the element, after completion of transposition, each replicon contains a copy of the element (top) and can thus, in turn, act as a donor.

As already mentioned, there are two major pathways to antibiotic resistance: mutational events in the chromosome and acquisition of foreign genes. Mutations can occur not only in a structural gene for the target of an antibiotic (as discussed for quinolones) but also in regulatory regions of genes (e.g., efflux pumps).

Resistance to an antibiotic is often inducible by the antibiotic itself. In this case, the drug should be considered as having two types of activities: induction of resistance and killing of the bacteria that act on distinct targets. Acquired resistance to vancomycin is a typical example of inducibility. Expression of the resistance genes of the *van* operon is controlled by a two-component regulatory system VanS/VanR, in which VanS acts as a sensor and VanR as a transcriptional regulator (**Figure 2**). In the presence of vancomycin in the environment, the signal is transduced from the sensor domain to the catalytic domain of VanS, leading to autophosphorylation of VanS followed by transfer of the phosphoryl group to the VanR response regulator. The phosphorylated regulator binds to the promoter regions resulting in transcriptional activation of the regulatory (*vanR*/*vanS*) and of the resistance genes, allowing expression of the resistance pathway (synthesis of modified peptidoglycan precursors) and elimination of the normal precursors ending in D-Ala-D-Ala. Under noninducing conditions, that is, in the absence of vancomycin, VanS acts as a phosphatase, dephosphorylates VanR resulting in arrest of expression of the resistance genes. Since antibiotic resistance usually corresponds to a gain of function, there is an associated biological cost resulting in the loss of fitness of the bacterial host. It therefore appears that modulation of gene expression probably reflects a good compromise between energy saving and adaptation to a rapidly changing environment.

Antibiotics Can Act as Pheromones

Antibiotics provide selective pressure for resistant bacteria to maintain and disseminate but they can also induce transfer of resistance genes. For example, it has been reported that (1) use of subinhibitory concentrations of penicillins increase the conjugal transfer of plasmid DNA from *E. coli* to *S. aureus* and *L. monocytogenes*, (2) oxacillin increased the frequency of *in vitro* transfer of Tn*916*, an enterococcal conjugative transposon, from *E. faecalis* to *Bacillus anthracis*, (3) transfer frequency of conjugative transposons belonging to the Tn*916*/Tn*1545* family, which contain a tetracycline resistance determinant, is increased 10- to 100-fold *in vitro* and *in vivo* in the presence of low concentrations of tetracycline, and (4) tetracycline also increases the transfer of a *Bacteroides* conjugative transposon.

Thus, several antibiotics can behave like pheromones: they are synthesized by specific cells (such as the *Actinomycetes* producers) and they act on another cell, at low concentrations, on very specific targets to promote DNA exchange.

Biological Cost of Antibiotic Resistance

The frequency of appearance of resistant strains in a bacterial population depends on several factors such as the volume of antibiotic used, the biological cost of resistance, and the ability of bacteria to compensate for the fitness cost. Acquisition of antibiotic resistance is often associated with a biological cost because (1) bacteria acquire a new gene or set of genes responsible for new functions, (2) the resistance mutations occur in genes with essential functions, or (3) of the replication and maintenance of extrachromosomal elements that bear the resistance genes. The biological cost allows determination of the stability and the potential reversibility of resistance. The fitness cost of antibiotic resistance could be assessed by measuring, in isogenic (susceptible and resistant) strains, the growth rate *in vitro* or in animals. A compensatory evolution could occur to reduce the biological cost, allowing the stabilization of the resistant bacteria in a natural population. This stabilization may allow resistant strains to compete with susceptible strains in an antibiotic-free environment. The compensatory evolution could be the result of (1) a true reversion of the resistance mutation or loss of the resistant element or (2) an acquisition of a second mutation (reverse mutation) located in the same (intragenic) or in another (extragenic) gene. Reversion mutations are more common than reversion to the susceptible phenotype. Once the resistant and compensated mutants are fixed in the bacterial population, a reversion to susceptibility is unlikely. Chances of reversibility of the resistance are also reduced in case of co-resistance to several antibiotics.

It was observed by competition experiments in *Helicobacter pylori* that clarythromicin resistance confers a biological cost in mice. Reduction of this cost, which was observed in clinical isolates, suggests that compensation is a clinically relevant phenomenon.

The biological fitness was also partially or totally restored in fusidic acid-resistant mutants of *S. aureus* and *Salmonella typhimurium* and in rifampin-resistant mutants of *E. coli*. Similar results were obtained in *S. enterica* that were resistant to a deformylase inhibitor, an antibiotic that targets peptide deformylase. Resistance mutations that occur in the *fmt* or *folD* gene confer a fitness cost in the absence of antibiotics. Intragenic mutations in the *fmt*/*folD* genes or extragenic mutations (such as amplification of genes encoding tRNAi) partially reduce the fitness cost.

Some resistant bacteria may have a normal growth suggesting that they have acquired a no-cost resistance mutation. A specific substitution in the *rpsL* gene (which encodes ribosomal protein S12), responsible for streptomycin resistance in several enteric bacteria, is a typical example of a no-cost high level resistance mutation.

Finally, the fitness cost of resistance depends on several factors such as the environmental conditions, the bacterial species, the specific resistance mutation, or the growth conditions. The methods used to determine the fitness cost are also crucial: one study demonstrates that no fitness cost was associated with vancomycin resistance in enterococci whereas another group found that vancomycin-susceptible enterococcal strains were more fit than their resistant counterparts.

Conclusion

Intrinsic or acquired resistance to antimicrobial drugs could be the result of different mechanisms. Resistance may (1) be limited to one class of antibiotics or (2) involve several classes by extended cross-resistance or co-resistance. Resistance determinants that are chromosomally located are vertically inherited whereas those that are part of mobile genetic elements can be vertically and horizontally acquired. There are three levels of exponential dissemination of antibiotic resistance: epidemics of resistant bacteria among mammals, resistance-plasmid epidemics due to the broad host range of conjugation, and gene epidemics among bacteria (transposons and integrons). Thus, resistance genes can easily disseminate under natural conditions. The biological cost associated with resistance, when it exists, is frequently reduced by a compensatory evolution that allows the stabilization of the resistant bacteria in the population. It is thus necessary to develop (1) strategies to reduce resistance dissemination (such as prudent use of antibiotics and increase of surveillance resistance) and (2) new antibiotics addressing novel targets and thus escaping from cross-resistance with already developed drugs. However, many *in vitro* and *in vivo* studies indicate that several pathways will confer, soon or later, resistance to every new antibiotic.

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Antifungal Agents

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The Polyenes
Griseofulvin
Cycloheximide
Pyrrolnitrin, Fenpiclonil, and Fludioxonil
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The Allylamines
The Benzylamines, Thiocarbamates, and Dithiocarbamates
The Benzimidazoles and Methylbenzimidazole Carbamates
The Morpholines
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The Echinocandins, Pneumocandins, and Papulocandins
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Glossary

emerging fungal infections Fungal infections caused by new or uncommon fungi.

granulocytopenia/neutropenia Acquired or chemically induced immunosuppression caused by low white blood cell counts.

immunocompromised Having a defect in the immune system.

in vitro *and* in vivo Describing or referring to studies carried out in the test tube and in animals, respectively.

mycoses and mycotic infections Diseases caused by yeasts or molds.

nephrotoxicity Damage to the kidney cells.

opportunistic infections Infections caused by saprophytic fungi or not true parasites.

Abbreviations

AMB	amphotericin B
CSF	cerebrospinal fluid
DMPC	dimyristoyl phosphatidylcholine
DMPG	dimyristoyl phosphatidylglycerol
GVHD	graft-versus-host disease
HSCT	hematopoietic stem cell transplant
MIC	mum inhibitory concentration
NYS	nystatin
OPC	oropharyngeal candidiasis

Defining Statement

Antifungal agents are naturally occurring or synthetically produced compounds that have *in vitro* or *in vivo* activity against yeasts, molds, or both. Fungi and mammalian cells are eukaryotes, and antifungal agents that inhibit synthesis of proteins, RNA, and DNA are potentially toxic to mammalian cells.

Introduction

Fungi can be unicellular (yeasts) and multicellular or filamentous (molds) microorganisms. Some medically important fungi can exist in each of these morphologic forms and are called dimorphic fungi. Of the estimated 250 000 fungal species described, fewer than 150 are known to be etiologic agents of disease in humans. Most fungi associated with disease are considered opportunistic pathogens (especially the yeasts) because they live as normal flora in humans, lower animals and plants, and rarely cause disease in otherwise healthy individuals. Many fungi, on the contrary, are important plant and lower animal parasites and can cause damage to crops (wheat rust, corn smut, etc.) and to fruit (banana wilt), forest (Dutch elm disease), and ornamental trees and other plants. Historically, the potato famine, which was the reason for the great migration from Ireland to the Americas, was caused by a fungal infection (potato blight). At the same time, fungi and their products play an important economic role in the production of alcohol, certain acids, steroids, antibiotics, and so on.

Because, the number of fungal diseases caused by both yeasts and molds has significantly increased during the past twenty years, especially among immunocompromised patients at high risk for life-threatening mycoses, 14 antifungal agents are currently licensed for the treatment and prevention of systemic fungal infections: the polyene amphotericin B and its three lipid formulations, the pyrimidine synthesis inhibitor 5-fluorocytosine (flucytosine), the imidazoles miconazole and ketoconazole, the triazoles fluconazole, itraconazole, voriconazole and posaconazole, and the echinocandins caspofungin, micafungin, and anidulafungin. However, the number of fungal diseases caused by both yeasts and molds has significantly increased during the past years, especially among the increased number of immunocompromised patients, who are at high risk for life-threatening mycoses. There are more agents for topical treatment as well as agriculture and veterinary use, and several agents are under investigation for the management of severe and refractory fungal infections in humans (**Table 1**).

This article summarizes the most relevant facts regarding the chemical structure, mechanisms of action and resistance, pharmacokinetics, safety, adverse interactions with other drugs and applications of the established systemic and topical antifungal agents currently licensed for clinical, veterinary, or agricultural uses. A shorter description is provided for antifungal compounds that are in the last phases of clinical development, under clinical trials in humans, or that have been discontinued from additional clinical evaluation. The former compounds have potential use as therapeutic agents. More detailed data regarding these agents are found in the references.

The Polyenes

The polyenes are macrolide molecules that target membranes containing ergosterol, which is an important sterol in the fungal cell membranes. Traces of ergosterol are also involved in the overall cell cycle of fungi.

Amphotericin B

Amphotericin B is the most important of the 200 polyenes. Amphotericin B replaced 2-hydroxystilbamidine in the treatment of blastomycosis in the mid-1960s. Two amphotericins (A and B) were isolated in the 1950s from *Streptomyces nodosus*, an aerobic bacterium, from a soil sample from Venezuela's Orinoco River Valley. Amphotericin B (the most active molecule) has seven conjugated double bonds, an internal ester, a free carboxyl group, and a glycoside side chain with a primary amino group (**Figure 1(a)**). It is unstable to heat, light, and acid pH. The fungistatic (inhibition of fungal growth) and fungicidal (lethal) activity of amphotericin B is due to its ability to combine with ergosterol in the cell membranes of susceptible fungi. Pores or channels are formed causing osmotic instability and loss of membrane integrity. This effect is not specific; and extends to mammalian cells. The drug binds to cholesterol, creating the high toxicity associated with all conventional polyene agents. A second mechanism of antifungal action has been proposed for amphotericin B, which is oxidation-dependent. Amphotericin B is highly protein bound (91–95%). Peak serum of 1–3 $\mu g\,ml^{-1}$ and trough concentrations of 0.5–1.1 $\mu g\,ml^{-1}$ are usually measured after the intravenous (i.v.) administration of 0.6 $mg\,kg^{-1}$ doses. Its half-life of elimination is 24–48 h, with a long terminal half-life of up to 15 days.

Although resistance to amphotericin is rare, quantitative and qualitative changes in the cell membrane sterols have been associated with the development of microbiological resistance both *in vitro* and *in vivo*. Clinically, resistance to amphotericin B has become an important problem, particularly with certain yeast and mold species, such as *Candida lusitaniae*, *Aspergillus terreus*, *Fusarium* spp., *Malassezia furfur*, *Pseudallescheria boydii* (*Scedosporium apiospermum*), *Scedosporium prolificans*, *Trichosporon beigelii*, and other emerging fungal pathogens.

The *in vitro* spectrum of activity of amphotericin B includes yeasts, dimorphic fungi, and most of the opportunistic molds. Clinically, amphotericin B has been considered as the gold standard antifungal agent for the management of most systemic and disseminated fungal infections and opportunistic mycoses. Although it penetrates poorly into the cerebrospinal fluid (CSF), amphotericin B is effective in the treatment of both *Candida* and *Cryptococcus* meningitis alone and/or in combination with 5-fluorocytosine. Toxicity is the limiting factor during amphotericin B therapy and has been classified as acute or delayed (**Table 2**). Nephrotoxicity is the most significant delayed adverse effect. Therefore, close monitoring of renal function tests, bicarbonate, electrolytes including magnesium, diuresis, and hydration status is recommended during amphotericin B therapy. Current recommendations regarding daily dosage, total dosage, duration, and its use in combination with other antifungal agents are based on the type of infection and the status of the host. Because severe fungal infections in the granulocytopenic host are difficult to diagnose and cause much mortality, empirical antifungal therapy with amphotericin B and other agents has improved patient care. Systemic prophylaxis for patients at high risk for invasive mycoses has also evolved. Amphotericin B adverse drug interactions can occur with the administration of electrolytes and other concomitant drugs. This drug is also used for the treatment of systemic infections in small animals, especially

Table 1 Antifungal agents, mechanisms of action, and their use

Antifungal class	*Antifungal target of action*	*Agent*	*Use*
Polyenes	Membranes containing ergosterol	Amphotericin B (AMB)	Systemic mycoses[a,b]
		Nystatin (NYS)	Superficial mycoses[a,b]
		AMB lipid complex	Systemic mycoses intolerant or refractory to AMB
		AMB colloidal dispersion	
		Liposomal AMB	
		Liposomal NYS	Under investigation
		Pimaricin	Topical keratitis[a]
Phenolic cyclohexane	Microtubule aggregation and DNA inhibition	Griseofulvin	Dermatophytic infections[a]
Natural glutarimide	Protein synthesis inhibition	Cycloheximide	Laboratory and agriculture
Phenylpyrroles	Unknown	Fenpiclonil	Agriculture
		Fludioxonil	
Synthetic pyrimidines	Fungal cytosine permease and deaminase	Flucytosine	Systemic (yeasts) in combination with AMB[a]
	Ergosterol inhibition	Triarimol	Agriculture
		Fenarimol	
Anilinopyrimidines	Enzyme secretion	Pyrimethanil	
		Cyprodinil	
Azoles	Ergosterol biosynthesis inhibition	**Imidazoles**	
		Clotrimazole	Topical, oral troche[a,b]
		Econazole	
		Isoconazole	
		Oxiconazole	
		Tioconazole	
		Miconazole	Topical and veterinary[b]
		Ketoconazole	Second-line drug for nonmeningeal systemic mycoses and veterinary[a,b]
		Enilconazole	Veterinary
		Epoxiconazole	Agriculture

(*Continued*)

Table 1 (Continued)

Antifungal class	*Antifungal target of action*	*Agent*	*Use*
		Fluquinconazole	
		Triticonazole	
		Prochoraz	
		Triazoles	
		Fluconazole	Candidiasis, cryptoccosis, coccidioimycosis, prophylaxis[b]
		Itraconazole	Candidiasis, aspergillosis, endemic mycoses, superficial diseases, long-term prophylaxis[a]
		Posaconazole	Prophylaxis (invasive *Aspergillus* and *Candida* infections), oropharyngeal candidiasis (OPC)
		Terconazole	Intravaginal
		Voriconazole	*Aspergillus* and *Candida* infections, salvage therapy (*S. apiospermum* and *Fusarium* infections)[a]
		Ravuconazole (BMS-207147, ER-30346)	Under investigation (phases II and III)
		Albaconazole (UR-9825)	Under investigation (phases I and II)
Allylamines		Terbinafine	Superficial infections
		Naftifine	Topical
Benzylamines		Butenafine	Topical
Thiocarbamates		Tolnaftate	Topical
		Tolciclate	
		Piritetrade	
Dithiocarbamates	Nonspecific	Mancozeb	Agriculture
		Thiram	
Benzimidazoles and methylbenzimidazole carbamates	Nuclear division	Carbendazim	Agriculture
		Benomyl	
		Thiophanate	
Morpholines	Ergosterol biosynthesis inhibition	Amorolfine	Topical
		Fenpropimorph	Agriculture
		Tridemorph	
Pyridines		Buthiobate	Agriculture
		Pyrifenox	

Echinocandins	Fungal (1,3)-glucan synthetase inhibition	Papulocandins	None
		Echinocandin B derivative	
		Pneumocandin derivatives	
		Anidulafungin	Invasive *Candida* infections, including candidemia[a]
		Caspofungin	Invasive *Candida* infections, including candidemia; empirical therapy (febrile neutropenic patients unresponsive to antibacterial therapy); salvage therapy (aspergillosis)[a]
		Micafungin	Invasive *Candida* infections, including candidemia Prophylaxis for HSCT patients and liver transplantation[a]
Pradimicins	Fungal saccharide (mannoproteins)	Pradamicin FA-2 (BMY 28864)	None
Benanomycins		Benanomycin A	Under investigation
Polyoxins	Fungal chitin synthase	Polyoxin D	None
Nikkomycins		Nikkomycin Z	Under investigation
Sordarins	Protein synthesis inhibition	GM 222712	Under investigation
		GM 237354	
Cinnamic acid	Cell wall	Dimethomorph	Agriculture
Oomycete fungicide	Oxidative phosphorylation	Fluazynam	Agriculture
Phthalimides	Nonspecific	Captan	Agriculture, horses (dermatophytic infections)
Cationic peptides	Lipid bilayer of biologimemberanes	Cecropin	None
		Indolicidin	
Synthetic peptides			Under investigation
Amino acid analogs	Amino acid synthesis interference	RI 331 Azoxybacillins Cispentacin	Under investigation
Candida isoleucyl-tRNA synthase inhibitor		Icofungipen	Phase II trials

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[a] Clinical and veterinary use; other applications for use in humans only.

[b] A human product used in veterinary.

Only licensed, commonly used, and antifungals under clinical investigation are listed; see text for other antifungals.

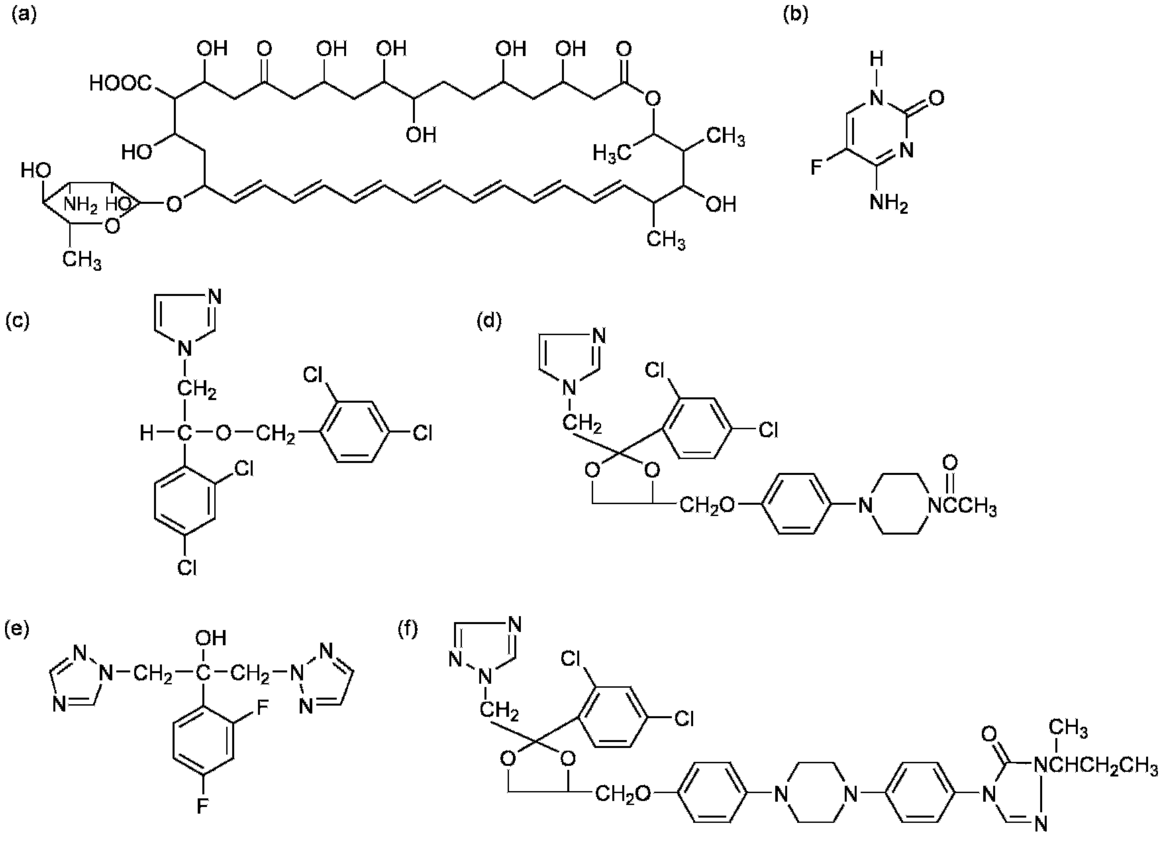

Figure 1 Chemical structures of some systemic licensed antifungal agents: (a) amphotericin B, (b) 5-fluorocytosine, (c) miconazole, (d) ketoconazole, (e) fluconazole, and (f) itraconazole.

blastomycosis in dogs, but it is not effective against aspergillosis. Side effects (especially in cats) and drug interactions are similar to those in humans.

Nystatin

Nystatin was the first of the polyenes to be discovered when it was isolated from *Streptomyces noursei* in the early 1950s. It is an amphoteric tetrane macrolide that has a similar structure (**Figure 2**(**a**)) and identical mechanism of action to those of conventional amphotericin B. Although it has an *in vitro* spectrum of activity similar to that of amphotericin B, this antifungal is used mostly for the therapy of gastrointestinal (orally) and mucocutaneous candidiasis (topically). This is not only due to its toxicity after parenteral administration to humans and lower animals but also to its lack of effectiveness when given i.v. to experimental animals. It is used for candidiasis in small animals and birds and for otitis caused by *Microsporum canis*.

Lipid Formulations

Amphotericin B lipid formulations

In an attempt to decrease the toxicity and increase the efficacy of amphotericin B in patients with deep-seated fungal infections, several lipid formulations of this antifungal have been developed since the 1980s. These preparations have selective toxicity or affinity for fungal cell membranes and theoretically promote the delivery of the drug to the site of infection while avoiding the toxicity of supramaximal doses of conventional amphotericin B. The result is a reduction of human erythrocytes lysis and higher doses of amphotericin B can be safely used. Three lipid formulations of amphotericin B have been evaluated in clinical trials: an amphotercin B lipid complex, an amphotericin B colloidal dispersion, and a liposomal amphotericin B. However, despite evidence of nephrotoxicity reduction, a significant improvement in their efficacy compared to conventional amphotericin B has not been demonstrated clearly. Although these three formulations have been approved for the treatment of invasive fungal infections that have failed conventional amphotericin B therapy, not enough information is available regarding their pharmacokinetics, drug interactions, long-term toxicities, and the differences in both efficacy and tolerance among the three formulations. Also, the most cost-effective clinical role of these agents as first-line therapies has not been elucidated.

Table 2 Adverse side effects of the licensed systemic antifungal agents

Side effect	*Drug*
Fever, chills	A, K, V, Fl, An
Rash	FC, K, I, FL, C, V, P
Nausea, vomiting	A, FC, K, I, FL, V, P, An
Abdominal pain	FC, K, V
Anorexia	A, K
Diarrhea	FC, V, M
Elevation of transaminases	FC, K, I, V, C, An (rare)
Hepatitis (rare)	FC, K, I, FL
Anemia	A, FC
Leukopenia, thrombocytopenia	FC, Fl, An (rare)
Decreased renal function (azotemia, acidosis, hypokalemia, etc.)	A, CV
Decreased testosterone synthesis	K (I, rare)
Adrenal insufficiency, menstrual irregularities, female alopecia	K
Syndrome of mineralocorticoid excess, pedal edema	I
Headache	A, FC, K, I, FL, V, An
Photophobia	K, V
Transient vision disturbances	V (30%)
Dizziness	I, V
Seizures	FL
Confusion	FC, V
Arthralgia, myalgia, thrombophlebitis	A
Ventricular tachycardia	C, V
Alopecia (rare)	Fl
Hypokalemia	Fl, C, V, An
Dyspnea and hypotension (rare)	An

Reproduced from Groll AH, Piscitelli SC, and Walsh TJ (1998) Clinical pharmacology of systematic antifungal agents: A comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. *Advances in Pharmacology* 44: 343–500 and Arikan S and Rex JH (2007) Antifungal agents. In: Murray, PR *et al.* (eds.) *Manual of Clinical Microbiology*, 9th ed. Washington, DC: ASM Press for more detailed information. A, amphotericin B; An, Anidulafungin; C, caspofungin; FC, flucytosine; K, ketoconazole; M, micafungin; Fl, fluconazole; I, itraconazole; V, voriconazole.

Liposomal amphotericin B

In the only commercially available liposomal formulation (ambisome), amphotericin B is incorporated into small unilamellar, spherical vesicles (60–70-nm liposomes). These liposomes contain hydrogenated soy phospatidylcholine and disteaoryl phosphatidylglycerol stabilized by cholesterol and amphotericin B in a 2:0.8:1:0.4 molar ratio. In the first liposomes, amphotericin B was incorporated into large, multilamellar liposomes that contained two phospholipids, dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG), in a 7:3 molar ratio (5–10% mole ratio of amphotericin B to lipid). This formulation is not commercially available, but it led to the development of commercial formulations.

Amphotericin B lipid complex

Amphotericin B lipid complex contains a DMPC/DMPG lipid formulation in a 7:3 ratio and a 50% molar ratio of amphotericin B to lipid complexes that form ribbon-like structures.

Amphotericin B colloidal dispersion

Amphotericin B colloidal dispersion contains cholesteryl sulfate and amphotericin B in a 1:1 molar ratio. This formulation is a stable complex of disk-like structures (122 nm in diameter and 4 nm thickness).

Liposomal nystatin

To protect human erythrocytes from nystatin toxicity, nystatin has been incorporated into stable, multilamellar liposomes, which contain DMPC and DMPG in a 7:3 ratio. Although it has been demonstrated that the efficacy of liposomal nystatin is significantly superior to that of conventional nystatin and is well tolerated in experimental murine models of systemic candidiasis and aspergillosis (fungal infections caused by *Candida* spp. and *Apergillus* spp.), evaluations in human subjects are limited.

Candicidin

Candicidin is a conjugated heptaene complex produced by *Streptomyces griseus* that is selectively and highly active *in vitro* against yeasts. It is more toxic for mammalian cells than either amphotericin B or nystatin; therefore, its use was restricted to topical applications for the treatment of vaginal candidiasis (infections by *Candida albicans* and other *Candida* spp.).

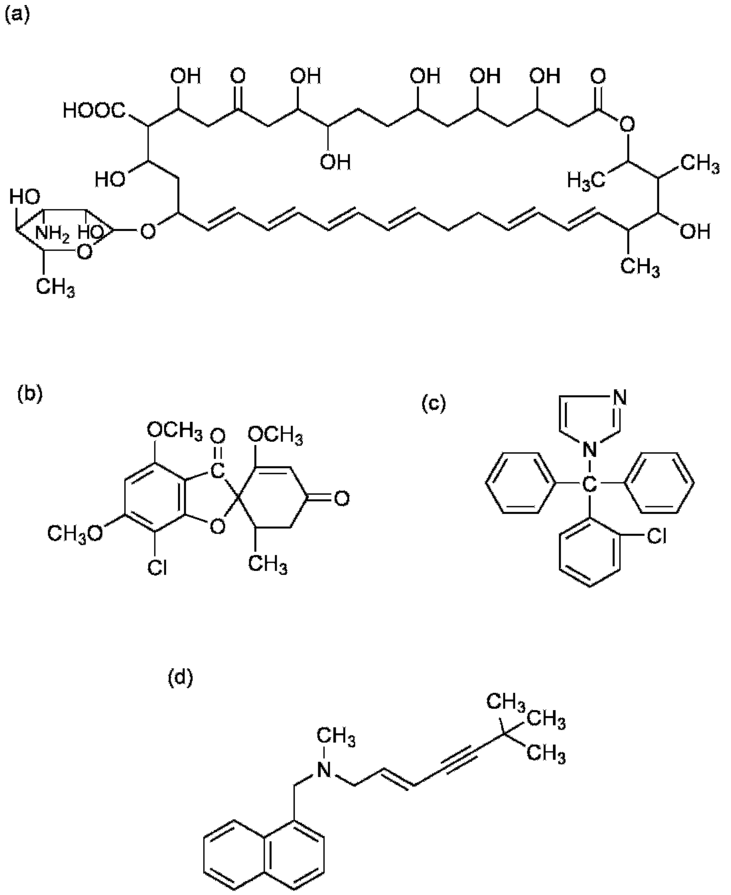

Figure 2 Chemical structures of the most commonly used topical antifungal agents: (a) nystatin, (b) griseofulvin, (c) clotrimazole, and (d) terbinafine.

Pimaricin

Pimaricin is a tetraene polyene produced by *Streptomyces natalensis*. It has a higher binding specificity for cholesterol than for ergosterol and, therefore, it is highly toxic for mammalian cells. The therapeutic use of pimaricin is limited to the topical treatment of keratitis (eye infections; also in horses) caused by the molds *Fusarium* spp., *Acremonium* spp., and other species.

Griseofulvin

Griseofulvin is a phenolic, benzyfuran cyclohexane agent (**Figure 2**(**b**)) that binds to RNA. It is a product of *Penicillium janczewski* and was the first antifungal agent to be developed as a systemic plant protectant. It acts as a potent inhibitor of thymidylate synthetase and interferes with the synthesis of DNA. It also inhibits microtubule formation and the synthesis of apical hyphal cell wall material. With the advent of terbinafine and itraconazole, the clinical use of griseofulvin as an oral agent for treatment of dermatophytic infections has become limited. However, it is frequently used for these infections in small animals, horses, and calves (skin only) as well as for equine sporotrichosis. Abdominal adverse side effects have been noted, especially in cats.

Cycloheximide

This is a glutaramide agent produced by *S. griseus*. This agent was among the three antifungals that were reported between 1944 and 1947. Although cycloheximide had clinical use in the past, it is currently used as a plant fungicide and in the preparation of laboratory media.

Pyrrolnitrin, Fenpiclonil, and Fludioxonil

Pyrrolnitrin is the fermentation product of *Pseudomonas* spp. It was used in the past as a topical agent. Fenpiclonil and fludioxonil (related to pyrrolnitrin) were the first of the phenylpyrrols to be introduced as fungicide for cereal seed.

The Synthetic Pyrimidines

5-Fluorocytosine (Flucytosine)

The synthetic 5-fluorocytosine is an antifungal metabolite that was first developed as an antitumor agent, but it is not effective against tumors. It is an oral, low-molecular-weight, fluorinated pyrimidine related to 5-fluorouracil and floxuridine (**Figure 1**(**b**)). It acts as a competitive antimetabolite for uracil in the synthesis of yeast RNA; it also interferes with thymidylate synthetase. Several enzymes are involved in the mode of action of 5-fluorocytosine. The first step is initiated by the uptake of the drug by a cell membrane-bound permease. Inside the cell, the drug is deaminated to 5-fluorouracil, which is the main active form of the drug. These activities can be antagonized *in vitro* by a variety of purines and pyrimidine bases and nucleosides. At least two metabolic sites are responsible for resistance to this compound. One involves the enzyme cytosine permease, which is responsible for the uptake of the drug into the fungal cell, and the other involves the enzyme cytosine deaminase, which is responsible for the deamination of the drug to 5-fluorouracil. Alterations of the genetic regions encoding these enzymes may result in fungal resistance to this drug by either decreasing the cell wall permeability or synthesizing molecules that compete with the drug or its metabolites.

5-Fluorocytosine has fungistatic but not fungicidal activity mostly against yeasts; its activity against molds is inoculum-dependent. Clinically, the major therapeutic role of 5-fluorocytosine is its use in combination with amphotericin B in the treatment of meningitis caused by the yeast *Cryptococcus neoformans*. The synergistic antifungal activity of these two agents has been demonstrated in clinical trials in non-HIV-infected and AIDS patients. 5-Fluorocytosine should not be used alone for the treatment of any fungal infections. The most serious toxicity associated with 5-fluorocytosine therapy is bone marrow suppression (6% of patients), which leads to neutropenia, thrombocytopenia, or pancytopenia (**Table 2**). Therefore, monitoring of the drug concentration in the patient's serum (serial 2-h levels post-oral administration) is highly recommended to adjust dosage and maintain serum levels between 40 and $60\,\mathrm{g\,ml^{-1}}$. Because the drug is administered in combination with amphotericin B, a decrease in glomerular filtration rate, a side effect of the latter compound, can induce increased toxicity to 5-fluorocytosine. Adverse drug interactions can occur with other antimicrobial and anticancer drugs, cyclosporine, and other therapeutic agents. Because of its toxic potential, 5-fluorocytosine should not be administered to pregnant women or animals. This drug has been used in combination with ketoconazole for cryptococcosis in small animals (very toxic for cats) and also for respiratory apergillosis and severe candidiasis in birds.

Triarimol, Fenarimol, Pyrimethanil, and Cyprodinil

Triarimol and fenarimol are pyrimidines with a different mechanism of action than that of 5-fluorocytosine. They inhibit lanosterol demethylase, an enzyme involved in the synthesis of ergosterol, which leads to the inhibition of this biosynthetic pathway. Triarimol and fenarimol are not used in medicine but are used extensively as antifungal agents in agriculture.

The anilinopyrimidines, pyrimethanil, and cyprodinil, inhibit the secretion of the fungal enzymes that cause plant cell lysis. Pyrimethanil has activity (without cross-resistance) against *Botrytis cinerea* (vines, fruits, vegetables, and ornamental plants) and *Venturia* spp. (apples and pears), whereas cyprodinil has systemic activity against *Botrytis* spp. but only a preventive effect against *Venturia* spp.

The Azoles

The azoles are the largest single source of synthetic antifungal agents; the first azole was discovered in 1944. As a group, they are broad spectrum in nature and mostly fungistatic. The broad spectrum of activity involves fungi (yeasts and molds), bacteria, and parasites. This group includes fused ring and N-substituted imidazoles and the N-substituted triazoles. The mode of action of these compounds is the inhibition of lanosterol demethylase, a cytochrome P450 enzyme.

Fused-Ring Imidazoles

The basic imidazole structure is a cyclic five-member ring containing three carbon and two nitrogen molecules. In the fused-ring imidazoles, two carbon molecules are shared in common with a fused benzene ring. Most of these compounds have parasitic activity (anthelmintic) and two have limited antifungal activity: 1-chlorobenzyl-2-methylbenzimidazole and thiabendazole.

1-Chlorobenzyl-2-methylbenzimidazole

The azole 1-chlorobenzyl-2-methylbenzimidazole was developed specifically as an anti-*Candida* agent. It has been used in the past in the treatment of superficial yeast and dermatophyte infections.

Thiabendazole

Thiabendazole was developed as an anthelmintic agent and has a limited activity against dermatophytes. It was also used in the past in the treatment of superficial yeast and dermatophytic infections. Thiabendazole has been used for aspergillosis and penicillosis in dogs.

N-Substituted (Mono) Imidazoles

In this group, the imidazole ring is intact and substitutions are made at one of the two nitrogen molecules. At least three series of such compounds have emerged for clinical and agricultural use. In the triphenylmethane series, substitutions are made at the nonsymmetrical carbon atom attached to one nitrogen molecule of the imidazole ring. In the second series, the substitutions are made at a phenethyl configuration attached to the nitrogen molecule. The dioxolane series is based on a 1,3-dioxolane molecule rather than on the 1-phenethyl molecule. These series vary in spectrum, specific level of antifungal activity, routes of administration, and potential uses.

Clotrimazole

Clotrimazole is the first member of the triphenylmethane series of clinical importance (**Figure 2(c)**). It has good *in vitro* activity at very low concentrations against a large variety of fungi (yeasts and molds). However, hepatic enzymatic inactivation of this compound, after systemic administration, has limited its use to topical applications (1% cream, lotion, solution, tincture, and vaginal cream) for superficial mycoses (nail, scalp, and skin infections) caused by the dermatophytes and *M. furfur*, for initial and/or mild oropharyngeal candidiasis (OPC; 10-mg oral troche), and for the intravaginal therapy (single application of 500-mg intravaginal tablet) of vulvovaginal candidiasis. Other intravaginal drugs require 3 to 7-day applications. This drug is also used for candidal stomatitis, dermatophytic infections, and nasal aspergillosis (infused through tubes) in dogs.

Bifonazole

Bifonazole is a halogen-free biphenylphenyl methane derivative. Bifonazole is seldom utilized as a topical agent for superficial infections, despite its broad spectrum of activity. Its limited use is the result of its toxic side effects for mammalian cells. Bifonazole is retained in the dermis for a longer time than clotrimazole.

Econazole, isoconazole, oxiconazole, and tioconazole

Other frequently used topical imidazoles include econazole (1% cream), isoconazole (1% cream), oxiconazole (1% cream and lotion), and tioconazole (6.5% vaginal ointment) (**Table 1**). As with clotrimazole, a single application of tioconazole is effective in the management of vulvovaginal candidiasis and as a nail lacquer for fungal onychomycosis (nail infections). Mild to moderate vulvovaginal burning has been associated with intravaginal therapy. Oxiconazole and econazole are less effective than terbinafine and itraconazole in the treatment of onychomycosis and other infections caused by the dermatophytes. Although topical agents do not cure onychomycosis as oral drugs do, they may slow down the spread of this infection. However, the recommended drugs for the treatment of onychomycosis are terbinafine (by dermatophytes) and itraconazole.

Miconazole

Miconazole (**Figure 1(c)**) was the first azole derivative to be administered intravenously for the therapy of systemic fungal infections. Clinically is only used as a topical agent for dermatophytic infections in humans and other large animals, fungal keratitis and pneumonia in horses, in birds, and aspergillosis in raptors. However, safety and efficacy data are not available (veterinary use).

Ketoconazole

Ketoconazole was the first representative of the dioxolane series (**Figure 1(d)**) to be introduced into clinical use and was the first orally active azole. Ketoconazole requires a normal intragastric pH for absorption. Its bioavailability is highly dependent on the pH of the gastric contents; an increase in pH will decrease its absorption, for example, in patients with gastric achlorhydria or treated with antacids or H_2-receptor antagonists (**Table 3**). This drug should be taken with either orange juice or a carbonated beverage. Ketoconazole pharmocokinetics corresponds to a dual model with an initial half-life of 1–4 h and a terminal half-life of 6–10 h, depending on the dose. This drug highly binds to plasma proteins and penetrates poorly into the CSF, urine, and saliva. Peak plasma concentrations of approximately 2, 8, and $20\,\mu g\,ml^{-1}$ are measured 1–4 h after corresponding oral doses of 200, 400, and 800 mg. The most common and dose-dependent adverse effects of nausea, anorexia, and vomiting (**Table 2**). They occur in 10% of the patients receiving a 400 mg dose and in approximately 50% of the patients taking 800 mg or higher doses. Another limiting factor of ketoconazole therapy is its numerous and significant adverse interactions with other concomitant drugs (**Table 3**).

In vitro, ketoconazole has a broad spectrum of activity compared to that of other azoles. However, due to its adverse side effects, its adverse interactions with other drugs, and the high rate of relapses, ketoconazole has been replaced by itraconazole and other triazoles. In noncancer patients, this drug can be effective in the treatment of superficial *Candida* and dermatophyte infections when the latter are refractory to griseofulvin therapy. Therapeutic failure with ketoconazole has been associated with low serum levels; monitoring of these levels is recommended in such failures. Ketoconazole also has been used for a variety of systemic and superficial fungal infections in cats and dogs.

Enilconazole

This is the azole most widely used in veterinary practice for the intranasal treatment of aspergillosis and

Table 3 Adverse interactions of the licensed systemic azoles with other drugs during concomitant therapy

Azole	*Concomitant drug*	*Adverse side effect of interaction*
K, FL, I	Nonsedating antihistamines, cisapride, terfenadine, astemizole	Fetal arrhythmia
K, Fl, I, V, C	Rifampin, isoniazid, phenobarbital, rifabutin, carbamazepine, and phenyton	Reduces azole or C plasma concentrations
K, Fl, I, V	Phenytoin, benzodiazepines, rifampin	Induces the potential toxicity levels of co-compounds
K, I	Antacids, H_2 antagonists, omeprazole, sucralfate, didanosine	Reduces azole absorption
K, Fl, I	Lovastin, simvastatin	Rhabdomyolysis
I	Indinavir, vincristine, quinidine, dicyclosporine, tacrolimus, methylprednisolone, and ritonavir	Induces potential toxicity co-comgoxin, compounds
Fl, I, V	Warfarin, rifabutin, sulfonylurea	Induces potential toxicity of co-compound
K	Saquinavir, chlordiazepoxide, methylprednisone	Induces potential toxicity of these compounds
K	Protein-binding drugs	Increases the release of fractions of free drug
K, C, V	Cyclosporine A	Nephrotoxicity
C, V	Tacrolimus	Tacrolimus can be decreased
C	Efavirenz, nevirapine, phenytoin, dexamethasone	C can be reduced (70 mg of C should be considered)
V	Omeprazole	Reduces omeprazole dose to one half
P	Phenytoin, cimetidine	Reduces P exposure
P	Cyclosporine A, tacrolimus	Increases concomitant drug exposure
P	Midazolam	
P	Rifabutin	Two-way interactions

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penicillosis as well as for dermatophytic infections. The side effects are few.

Epoxiconazole, fluquinconazole, triticonazole, and prochloraz

Epoxiconazole, fluquinconazole, and triticonazole are important agricultural fungicides, which have a wider spectrum of activity than that of the earlier triazoles, triadimefon, and propiconazole, and the imidazole, prochloraz, as systemic cereal fungicides. However, development of resistance to these compounds has been documented.

The Triazoles

The triazoles are characterized by a more specific binding to fungal cell cytochromes than to mammalian cells due to the substitution of the imidazole ring by the triazole ring. Other beneficial effects of this substitution are (1) an improved resistance to metabolic degradation, (2) an increased potency, and (3) a superior antifungal activity. Several triazoles are currently licensed for antifungal systemic therapy and other triazoles are at different levels of clinical evaluation (**Table 1**).

Fluconazole

Fluconazole is a relatively small molecule (**Figure 1(e)**) that is partially water soluble, minimally protein bound, and excreted largely as an active drug in the urine. It penetrates well into the CSF and parenchyma of the brain and the eye, and it has a prolonged half-life (up to 25 h in humans). Its pharmacokinetics are linear and independent on the route of administration and the drug formulation. Fluconazole is well absorbed orally (its total bioavailability exceeds 90%), and its absorption is not affected by food or gastric pH. Plasma concentrations of 2–7 $\mu g\,ml^{-1}$ are usually measured in healthy subjects after single doses of 100 and 400 mg. After multiple doses, the peak plasma levels are 2.5 times higher than those of single doses. The CSF to serum fluconazole concentrations are between 0.5 and 0.9% in both healthy human subjects and laboratory animals.

Fluconazole does not have *in vitro* or *in vivo* activity against most molds. Both oral and i.v. formulations of fluconazole are available for the treatment of candidemia in nonneutropenic and other nonimmunosuppressed patients, mucosal candidiasis (oral, vaginal, and esophageal), and chronic mucocutaneous candidiasis in patients of all ages. Fluconazole is the current drug of choice for maintenance therapy of AIDS-associated cryptococcal and coccidioidal meningitis. However, since resistance to this drug can develop during therapy, fluconazole prophylaxis should be reserved for HIV-infected individuals or AIDS patients, or for patients with prolonged (2 weeks) and profound neutropenia (500 cells). Although the recommended dosage of fluconazole for adults is 100–400 mg each day (q.d.) higher doses (800 mg q.d.) are required for the treatment of severe invasive infections and for infections caused by a *Candida* spp. that exhibit a minimum inhibitory concentration (MIC) of 8 μg ml^{-1}. Due to fluconazole resistance among *Candida krusei* (intrinsically resistant) and *Candida glabrata* ($\sim$15% resistant) isolates, fluconazole use for the treatment of such infections is precluded. In contrast to the imidazoles and itraconazole, fluconazole does not exhibit major toxicity side effects (2.8–16%). However, when the dosage is increased above 1200 mg, adverse side effects are more frequent (**Table 2**). Fluconazole interactions with other concomitant drugs are similar to those reported with other azoles, but they are less frequent than those exhibited by ketoconazole and itraconazole (**Table 3**). Fluconazole has been used to treat nasal aspergillosis and penicillosis in small animals and birds when topical enilconazole is not feasible.

Itraconazole

Oral and i.v. itraconazole are commercially available for the treatment of certain systemic mycoses. In contrast to fluconazole, itraconazole is insoluble in aqueous fluids; it penetrates poorly into the CSF and urine but well into skin and soft tissues; and it is highly protein bound (90%). Its structure is closely related to that of ketoconazole (**Figure 1(f)**), but itraconazole has a broader spectrum of *in vitro* and *in vivo* antifungal activity. Similar to ketoconazole, itraconazole is soluble only at low pH and is better absorbed when the patient is not fasting. Absorption is erratic in cancer patients or when the patient is taking concomitant H_2-receptor antagonists, omeprazole, or antacids. Therefore, this drug should be taken with food and/or acidic fluids. Plasma peak (1.5–4 h) and trough concentrations between 1 and 2.2 μg ml^{-1} and 0.4 and 1.8 μg ml^{-1}, respectively, are usually obtained after 200-mg dosages (capsule) as either single daily dosages (per orally (p.o.) or twice daily (b.i.d.)) or after i.v. administration (b.i.d.) for 2 days and q.d. for more days; these concentrations are also obtained in cancer patients receiving 5 mg kg^{-1} divided into two oral solution dosages.

Clinically, itraconazole (200–400 mg/day) supplanted ketoconazole as therapy for endemic, non-life-threatening mycoses including unresponsive cases to other azoles. For more severe mycoses, higher doses are recommended and clinical resistance may emerge. The oral solution is better absorbed than the tablet and has become useful for the treatment of HIV-associated oral and esophageal candidiasis. However, monitoring of itraconazole plasma concentrations is recommended during treatment of both superficial and invasive diseases: Drug concentration 0.5 μg ml^{-1} by high performance liquid chromatography and 2 μg ml^{-1} by bioassay appear to be critical for favorable clinical response. Treatment with itraconazole has been associated with less adverse and mostly transient side effects (10%) than that with ketoconazole (**Table 2**), and these effects are usually observed when the patient takes up to 400 mg during several periods of time. In animals, itraconazole has been used for the treatment of aspergillosis, crytococcosis, blastomycosis (especially in dogs), equine sporotrichosis, and osteomyelitis (caused by *Coccidiodis immitis* in large animals), but its use is minimal. No data are available regarding its side effects or drug interactions in animals.

Voriconazole (UK-109496)

Voroconazole is a triazole related to fluconazole obtained by replacement of one triazole moiety by fluoropyrimidine and α-methylation groups (**Figure 3(a)**); it is insoluble in aqueous fluids. As do the other azoles, voriconazole acts by inhibiting fungal cytochrome P450-dependent, 14-α-sterol demethylasemediated synthesis of ergosterol. Voriconazole pharmacokinetics in humans are nonlinear and dosedependent. Following single oral doses, peak plasma concentrations were achieved after 2 h and multiple doses resulted in a higher (8 times) accumulation. The mean half-life of elimination is about 6 h. Voriconazole binds to proteins (65%), is extensively metabolized in the liver (80%), and is found in the urine (78–88%) practically unchanged after a single dose. Voriconazole has good oral bioavility, distributes widely into tissues including those of the central nervous system. Voriconazole has an improved *in vitro* fungistatic activity and an increased potency against most fungi compared to those of fluconazole. It is fungicidal against some fungi, especially *Aspergillus* spp. It does not have activity against the Zygomycetes, certain isolates of *Candida rugosa*, *Sporothrix schenckii* and a significant number of *Rhodotorula* strains, especially *R. muciloginosa*. Clinically, it has been approved for the primary treatment of invasive aspegillosis, salvage therapy for other mould infections caused by *S. apiospermum* and *Fusarium* spp., candidemia and other infections caused by *Candida* spp. The drug is generally well tolerated with only few side effects, for example, hepatic (10–15%), transient visual (10–15%) and skin rash (1–5%; see **Table 2**). For reported drug interactions see **Table 3**.

Posaconazole (SCH 56592)

Posaconazole is the product of a modification of the n-alkyl side chain of SCH 51048, which included a variety of chiral substituents (**Figure 3(b)**). Posaconazole absorption from the intestinal tract is slow and peak serum concentrations are achieved 11–24 h after the actual dose. Posaconazole exhibits dose-proportional absorption up to 800 mg; dividing the dose and food or liquid supplements increase exposure. It has a large volume distribution (1774 l) and it is highly protein bound (>98%). Posaconazole has a prolonged elimination half-life (20–66 h); it is not extensively metabolized (approximately 14%) and is eliminated unchanged in the feces (66%). The *in vitro* fungistatic and

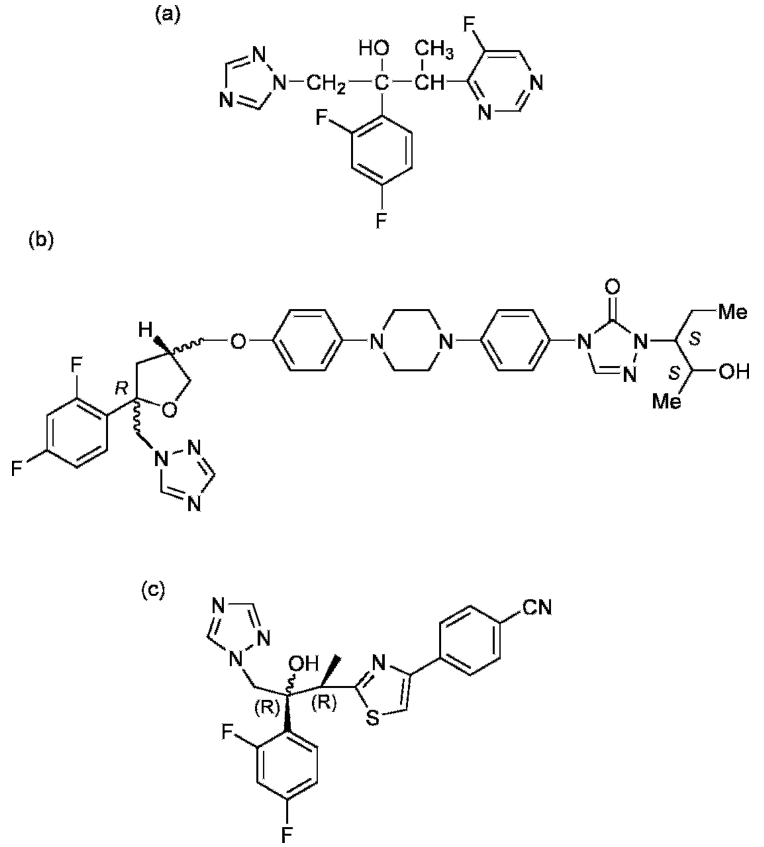

Figure 3 Chemical structures of three triazoles: (a) voriconazole, (b) posaconazole, and (c) ravuconazole (BMS-207147; ER-30346).

fungicidal activities of posaconazole are similar to those of voriconazole and amphotericin B against yeasts, the dimorphic fungi, most opportunistic molds including the Zygomycetes, certain phaeoid fungi, and the dermatophytes.

Oral posaconazole has been approved for prophylaxis of invasive *Aspergillus* and *Candida* infections in patients at high risk due to being severely immunocompromised hematopoietic stem cell transplant (HSCT) recipients with graft-versus-host disease (GVHD) or those with hematologic malignancies with prolonged neutropenia from chemotherapy. It also has been approved for the treatment of OPC.

Inducers of the UDP glucuronidation pathway may affect posaconazole plasma concentrations (**Table 3**). Posaconazole is generally well tolerated and serious adverse effects (most common, altered drug level, increased hepatic enzymes, nausea, rash, and vomiting) have occurred in 8% of patients (**Table 2**).

Terconazole

Terconazole was the first triazole marketed for the topical treatment of vaginal candidiasis and superficial dermatophyte infections. Currently, it is only used for vulvovaginal candidiasis (0.4 and 0.8% vaginal creams and 80 mg vaginal suppositories).

Investigational Triazoles

Other triazoles are currently under clinical investigation and are at earlier stages of development (**Table 1**). Triazoles such as saperconazole (R *66905*), BAY R 8783, SCH 39304, SCH 51048, BAY 3783, and SDZ 89-485 were discontinued from further development due a variety of adverse side effects.

Ravuconazole (BMS-207147; ER-30346)

BMS-207147 is a novel oral thiazole-containing triazole (**Figure 3**(**c**)) with a broad spectrum of activity against the majority of opportunistic pathogenic fungi. Ravuconazole has a similar or superior *in vitro* activity compared to those of the agents against most pathogenic yeasts, with the exceptions of *Candida tropicalis* and *C. glabrata*. It also has good *in vivo* antifungal activity in murine models for the treatment of invasive aspergillosis, candidiasis, and cryptococcosis. Ravuconazole shows good pharmacokinetics in animals that is similar to that of itraconazole. This indicates that it is absorbed at levels comparable to those of itraconazole. However, the half-life ravuconazole (4 h) is longer than that of itraconazole (1.4 h) and similar to that of fluconazole. The potential use of ravuconazole has yet to be determined in clinical trials in humans (phases II–III trials).

D 0870

Although more *in vitro* and *in vivo* studies were conducted with D 0870 than with SDZ-89-485, and D 0870 showed good antifungal activity, this drug was also discontinued by its original developers. The *in vitro* activity of D 0870 is lower than that of itraconazole against *Aspergillus* spp., but higher for the common *Candida* spp. Therefore, evaluation of this compound has been continued by another pharmaceutical company for the treatment of OPC in HIV-infected individuals. It has also shown activity against the parasite *Trypanosoma cruzi*.

T-8581

T-8581 is a water-soluble 2-fluorobutanamide triazole derivative. High peak concentrations (7.14–12 $\mu g\,ml^{-1}$) of T-8581 were determined in the sera of laboratory animals following the administration of single oral doses of 10 $mg\,kg^{-1}$ and the drug was detected in the animals sera after 24 h. The halflife of T-8581 varies in the different animal models from 3.2 h in mice to 9.9 h in dogs. Animal studies suggest that the absorption of this compound is almost complete after p.o. dosages. The maximum solubility of T-8581 is superior (41.8 $mg\,ml^{-1}$) to that of fluconazole (2.6 $mg\,ml^{-1}$), which suggests the potential use of this compound as an alternative to fluconazole for high-dose therapy.

T-8581 has shown potent *in vitro* antifungal activity against *Candida* spp., *C. neoformans*, and *Aspergillus fumigatus*. The activity of T-8581 is similar to that of fluconazole for the treatment of murine systemic candidiasis and superior to itraconazole for aspergillosis in rabbits. The safety of T-8581 is under evaluation.

UR-9746, UR-9751, and albaconazole (UR-9825)

UR-9746, UR-9751, and albaconazole are similar fluoridated triazoles that contain an N-morpholine ring. The pharmacokinetics of these compounds in laboratory animals has demonstrated peak concentrations (biological activity) of 184 (UR-9746) and 34 $\mu g\,ml^{-1}$ (UR-9751) after 8 and 8–24 h, respectively. *In vitro* and *in vivo* activity has been demonstrated with these compounds against *Candida* spp., *C. neoformans*, *Aspergillus* spp., and other molds. These antifungals lacked detectable toxicity in experimental animal infections. Albaconazole is currently in phases I–II trials.

TAK 187, SSY 726, and KP-103

Some *in vitro* and very little *in vivo* data are available for these new triazoles.

The Allylamines

The allylamines act by inhibiting squalene epoxidase, which results in a decrease in the ergosterol content and an accumulation of squalene.

Terbinafine and Naftifine

Terbinafine (**Figure 2(d)**) is the most active derivative of this class of antifungals. It has an excellent *in vitro* activity against the dermatophytes and other filamentous fungi, but its *in vitro* activity against the yeasts is controversial. It follows linear pharmacokinetics over a dose range of 125–750 mg; drug concentrations of 0.5–2.7 $\mu g\,ml^{-1}$ are detected 1 or 2 h after a single oral dose. Terbinafine has replaced griseofulvin and ketoconazole for the treatment of onychomycosis and other infections caused by dermatophytes (oral and topical). It is also effective for the treatment of vulvovaginal candidiasis. It is usually well tolerated at oral doses of 250 and 500 mg per day and the side effects (10%) are gastrointestinal and cutaneous. The metabolism of terbinafine may be decreased by cimetidine and increased by rifampin.

Naftifine

Pharmacokinetics and poor activity have limited the use of naftifine to topical treatment of dermatophytic infections.

The Benzylamines, Thiocarbamates, and Dithiocarbamates

The benzylamine, butenafine, and the thiocarbamates, tolnaftate, tolciclate, and piritetrade, also inhibit the synthesis of ergosterol at the level of squalene. Their clinical use is limited to the topical treatment of superficial dermatophytic infections. The Bordeaux mixture (reaction product of copper sulfate and lime) was the only fungicide used until the discovery of the dithiocarbamate fungicides in the mid-1930s. Of those, mancozeb and thiram are widely used in agriculture, but because they are only surface-acting materials frequent spray applications are required. Ferbam, maneb, and zineb are not used as much.

The Benzimidazoles and Methylbenzimidazole Carbamates

A great impact on crop protection was evident with the introduction of the benzimidazoles and other systemic (penetrate the plant) fungicides. These compounds increased spray intervals to 14 days or more. The methylbenzimidazole carbamates (MBCs; carbendazim, benomyl, and thiophanate) inhibit nuclear division and are also systemic agricultural fungicides. However, since MBC-resistant strains of *B. cinerea* and *Penicillium expansum* have been isolated, these compounds should be used in combination with *N*-phenylcarbamate or agents that have a different mode of action.

The Morpholines

The morpholines interfere with 14 reductase and 7,8 isomerase enzymes in the ergosterol biosynthetic pathway, which leads to an increase in toxic sterols and an increase in the ergosterol content of the fungal cell.

Amorolfine

Amorolfine, a derivative of fenpropimorph, is the only morpholine that has a clinical application for the topical treatment of dermatophytic infections and candidal vaginitis.

Fenpropimorph, Tridemorph, and Other Morpholines

Protein binding and side effects have precluded the clinical use of these morpholines, but they are important agricultural fungicides.

The Pyridines

The pyridines are another class of antifungal agents that inhibit lanosterol demethylase.

Buthiobate and Pyrifenox

These agents are important agricultural fungicides.

The Echinocandins, Pneumocandins, and Papulocandins

The echinocandins and papulocandins are naturally occuring metabolites of *Aspergillus nidulans* var. *echinulate* (echinocandin B), *A. aculeatus* (aculeacin A), and *Papularia sphaerosperma* (papulocandin). They act specifically by inhibiting the synthesis of fungal (1,3)-glucan synthetase, which results in the depletion of glucan, an essential component of the fungal cell wall.

The Papulocandins

The papulocandins A–D, L687781, BU4794F, and chaetiacandin have *in vitro* activity only against *Candida* spp. but poor *in vivo* activity, which precluded clinical development.

The Echinocandins and Pneumocandins

The echinocandins include echinocandins, pneumocandins, aculeacins, mulundo- and deoxymulundocandin, sporiofungin, vWF 11899 A–C, and FR 901379. The echinocandins have better *in vitro* and *in vivo* antifungal activity than the papulocandins. Pharmaceutical development has resulted in several semisynthetic echinocandins with an improved antifungal activity compared to those of the naturally occurring molecules described previously.

The pneumocandins have similar structures to those of the echinocandins, but they possess a hexapeptide core with a β-hydroxyglutamine instead of the threonine residue, a branched-chain ^{14}C fatty acid acyl group at the N-terminus, and variable substituents at the C-terminal proline residue. The pneumocandins are fermentation products of the mold *Zalerion arboricola*. Of the three naturally occurring pneumocandins (A–C), only A and B have certain antifungal activity *in vitro* and *in vivo* against *Candida* spp. and *Pneumocystis carinii* (in rodents), but they are nonwater-soluble.

Cilofungin (LY121019)

Cilofungin is a biosemisynthetic analog of the naturally occurring and toxic (erythrocyteslysis) 4-*n*-octyloxybenzoyl-echinocandin B. Although it showed good *in vitro* activity against *Candida* spp., this drug was discontinued owing to the incidence of metabolic acidosis associated with its i.v. carrier, polyethylene glycol.

Anidulafungin (VER-002; LY303366, V-echinocandin)

This is another semisynthetic cyclic lipopeptide which resulted from an increase of aromatic groups in the cilofungin sidechain (**Figure 4(a)**). It has high potency and oral and parental bioavailability. In laboratory animals, peak levels in plasma (5 or 6 h) of 0.5–2.9 μg ml^{-1} have been measured after single doses of 50–250 mg kg^{-1}. In humans, peak levels of 105–1624 ng ml^{-1} are measured after oral administrations of 100–1000 mg kg^{-1}. Its pharmacokinetics are linear and is characterized by a short distribution half-life (0.5–1 h) and a volume distribution of 30–50 l. The terminal elimination half-life is about 40–50 h and its clearance about 1 l/hr. Tissue concentrations are usually higher than those in plasma in animals. Systemic exposures of anidulafungin (post i.v. doses) are dose proportional. Anidulafungin is moderately protein bound in humans (84%).

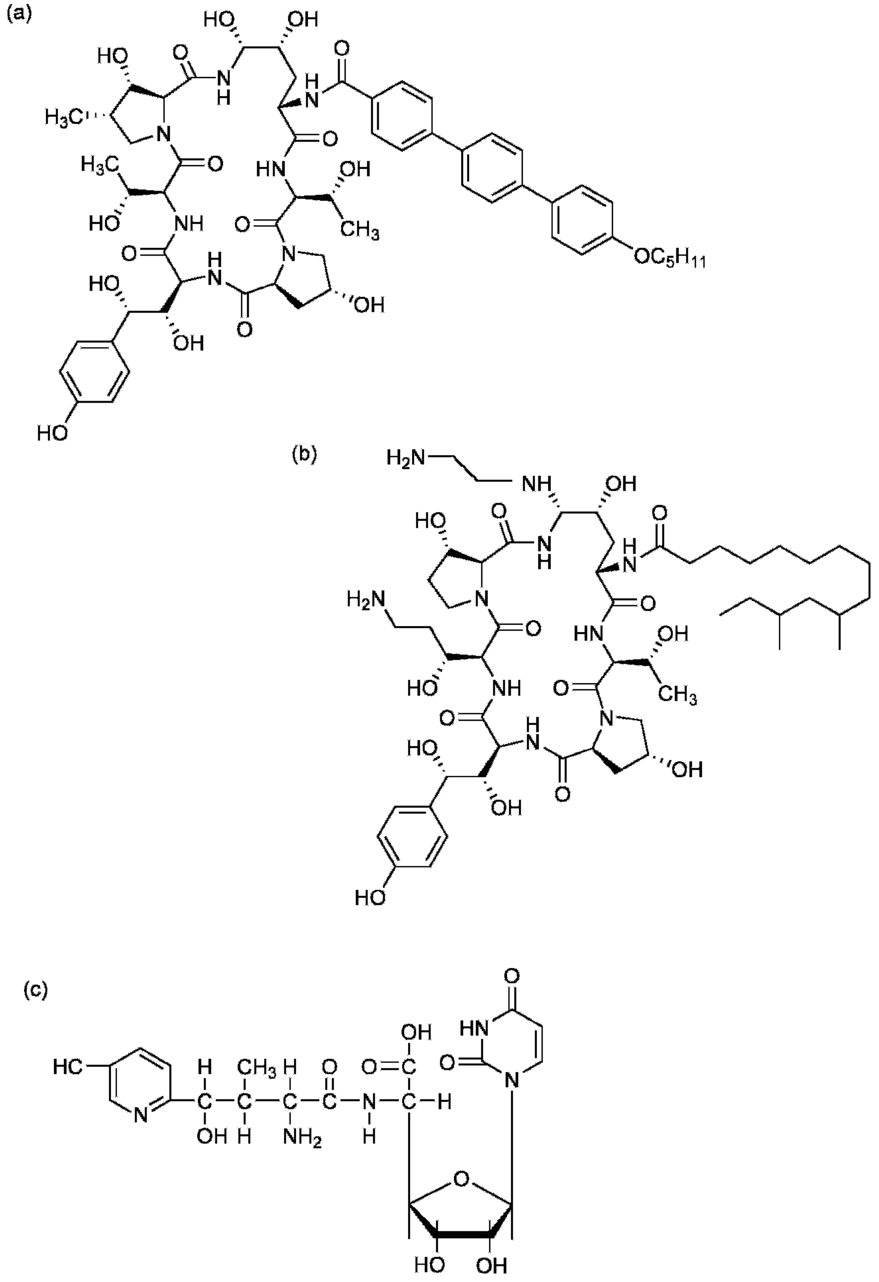

Figure 4 Chemical structures of anidulafungin (VER-002; V-echinocandin LY303366), caspofungin (L 743872 or MK-0991), and nikkomycin Z.

Anidulafungin has good *in vitro* activity against a variety of yeasts, including isolates resistant to itraconazole and fluconazole, and molds. This compound is not active against *C. neoformans* and *T. beigelii*; its MICs for certain molds are higher than those of the azoles, but its fungicidal activity against some species of *Candida* is superior to those agents. Anidulafungin has shown oral efficacy in animal models of systemic candidiasis and pneumocystis pneumonia. Anidulafungin has been approved for the treatment of candidemia (200 mg loading dose on first day followed by 100 mg daily dose) and other candidal infections (100 mg loading dose on first day followed by 50 mg daily dose). Laboratory abnormalities in liver functions have been observed, but no clinically relevant drug–drug interactions have been observed with drugs likely to be coadministered with anidulafungin.

L693989, L733560, L705589, and L731373

Modification of the original pneumocandin B by phosphorylation of the free phenolic hydroxyl group led to the improved, water-soluble pneumocandin B phosphate (L693989). Further modifications of pneumocandin B led to the water-soluble semisynthetic molecules L733560, L705589, and L731373. Although studies were conduced in laboratory animals, these molecules were not evaluated in humans.

Caspofungin (MK-0991 or L743872)

Caspofungin (**Figure 4**(**b**)) is the product of a modification of L733560 and was selected for evaluation in clinical trials in humans. Caspofungin is water soluble. As are the other semisynthetic pneumocandins. Caspofungin is highly protein bound (97%) with a half-life that ranges from 5 to 7.5 h and drug concentrations are usually higher in tissue than in plasma. As for anidulafungin and micafungin, caspofungin exhibits favorable dosedependent linear pharmacokinetics and only i.v. formulations are available. Caspofungin has fungistatic and fungicidal activities similar to those of anidulafungin against most *Candida* spp. and lower activity against the dimorphic fungi. It also has fungistatic *in vitro* activity against some of the other molds, especially *Aspergillus* spp. The drug is not effective for the treatment of disseminated experimental infections caused by *C. neoformans*. In laboratory animals, the drug is mostly well tolerated, but histamine release, mild hepatotoxicity, fever, nausea, vomiting, and rash have been reported (**Table 2**). In humans, caspofungin (50 mg) has been approved for the treatment of systemic candidiasis and other *Candida* infections, refractory invasive aspergillosis or for patients intolerant to other agents and for empiric therapy for febrile neutropenic patients unresponsive to antibacterial therapy.

Micafungin (FK 463)

Micafungin is a semisynthetic derivative of a naturally occurring lipoprotein that was synthesized by a chemical modification from a product of the mould *Coleophora impedir*. As the other echinocandins, it has *in vitro* activity against *Candida* and *Aspergillus* species, but is inactive against *C. neoformas*, *T. beigelii*, and *Fusarium* spp. The drug is well tolerated (**Table 2**). It is protein binding (99%) and plasma concentrations attain a steady state by day 4 with repeated doses. Micafungin has recently been approved for the treatment of patients with esophageal invasive (including candidemia) candidiasis and the prophylaxis of *Candida* infections in patients undergoing HSCT.

The Pradimicins and Benanomycins

The pradimicins and benanomycins are fungicidal metabolites of the *Actinomycetes*, but several semi-synthetic molecules have also been produced. They act by disrupting the cell membrane through a calcium-dependent binding with the saccharide component of mannoproteins, which results in disruption of the plasma membrane and leakage.

Pradimicin A (BMY 28567) and FA-2 (BMY 28864)

The poor solubility of pradimicin A led to the development of BMY 28864, which is a water-soluble derivative of pradimicin FA-2. BMY 28864 appears to have good *in vitro* and *in vivo* activity against most common yeasts and *A. fumigatus*. Clinical trials in humans have not been conducted.

BMS 181184

This compound is either a semisynthetic or biosynthetic derivative of BMY 28864. Although it was selected for further clinical evaluation due its promising *in vitro* and *in vivo* data, elevation of liver transaminases in humans led to the discontinuation of this drug.

Benanomycin A

This compound has shown the best antifungal activity among the various benanomycins. Its great advantage compared to other new antifungals is its good *in vivo* activity in animals against *P. carinii*.

The Polyoxins and Nikkomycins

The polyoxins are produced by *Streptomyces cacao* and the nikkomycins by *Streptomyces tendae*. The former compounds were discovered during a search for new agricultural fungicides and pesticides. Both polyoxins and nikkomycins are pyrimidine nucleosides that inhibit the enzyme chitin synthase, which leads to the depletion of chitin in the fungal cell wall. These molecules are transported into the cell via peptide permeases.

Polyoxin D

Although this compound has *in vitro* antifungal activity against *C. immitis* (parasitic phase), *C. albicans*, and *C. neoformans*, it was not effective in the treatment of systematic candidiasis in mice.

Nikkomycin Z

This compound (**Figure 4**(**c**)) appears to have both *in vitro* and *in vivo* activity against *C. immitis*, *Blastomyces dermatitidis*, and *Histoplasma capsulatum* as well as *in vitro* activity against *C. albicans* and *C. neoformans*. Clinical trials are pending.

The Sordarins

The natural sordarin GR 135402 is an antifungal fermentation product of *Graphium putredinis*. The compounds GM 193663, GM 211676, GM 222712, and GM 237354 are synthetic derivatives of GR 135402. *In vitro*, GM 222712 and GM 237354 have shown broad-spectrum antifungal activity for a variety of yeasts and molds. Clinical trials are pending.

Dimethomorph and Fluazinam

Dimethomorph is a cinnamic acid derivative for use against *Plasmopara viticola* on vines and *Phytophthora infestans* on tomatoes and potatoes; it is not cross-resistant to phenylamides (systemic controllers of *Phycomycetes* plant infections). Fluazinam is used in vines and potatoes but also acts against *B. cinera* as an uncoupler of oxidative phosphorylation.

The Phthalimides

The discovery of captan in 1952 and later of the related captafol and folpet initiated the proper protection of crops by the application of specific fungicides. Captan is also used to treat dermatophytic infections in horses and cattle, but it causes skin sensitization in horses.

Other Antifungal Approaches

Natural and Synthetic Cationic Peptides

Cationic peptides provide a novel approach to antifungal therapy that warrants further investigation.

Cecropin

Cecropin is a natural lytic peptide that is not lethal to mammalian cells and binds to ergosterol. Its antifungal activity varies according to the fungal species being challenged.

Indolicidin

Indolicidin is a tridecapeptide that has good *in vitro* antifungal activity and when incorporated into liposomes has activity against experimental aspergillosis in animals.

Synthetic peptides

Synthetic peptides have been derived from the natural bactericidal-permeability increasing factor. They appear to have *in vitro* activity against *C. albicans*, *C. neoformans*, and *A. fumigatus* and also show synergistic activity with fluconazole *in vitro.*

Amino Acid Analogs

RI 331, the azoxybacillins, and cispentacin are amino acid analogs with good *in vitro* antifungal activity against *Aspergillus* spp. and the dermatophytes (RI 331 and azoxybacillins) and also good *in vivo* activity (cispentacin). RI 331 and the azoxybacillins inhibit homoserine dehydrogenase and the biosynthesis of sulfur-containing amino acids, respectively.

Icofungipen (BAY 10-8888 and PLD-118)

It is a *Candida* isoleucyl-tRNA synthase inhibitor that is currently in phase II trials.

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Antiviral Agents

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Defining Statement
Introduction
Therapeutics for Herpesvirus Infections
Therapeutics for Respiratory Virus Infections
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Glossary

alanine aminotransferase An enzyme found in the liver and blood serum, the concentration of which is often elevated in cases of liver damage.

antiretroviral agent Any drug used in treating patients with human immunodeficiency virus (HIV) infection.

antiviral resistance The developed resistance of a virus to a specific drug.

bilirubin A greenish compound formed in the liver from the degradation of the hemoglobin from degraded red blood cells.

bioavailability The property of a drug to be absorbed and distributed within the body in a way that preserves its useful characteristics; for example, it is not broken down, inactivated, or made insoluble.

chemoprophylaxis Preventive treatment with chemical agents such as drugs.

codon A triplet of three consecutive nucleotide components in the linear genetic code in DNA or messenger RNA, which designates a specific amino acid in the linear sequence of a protein molecule.

creatinine An end product of energy metabolism found in the blood in uniform concentration, which is excreted by the kidney at a constant rate. Alterations of this rate are considered an indication of kidney malfunction.

cytokine One of a variety of proteins which has a regulatory effect on a cell.

drug resistance Decreased susceptibility to antiviral usually due to changes in the amino acid residues of target enzyme. For example, a substitution of valine for methionine at residue 184 of the reverse transcriptase enzyme of HIV confers resistance to lamivudine (M184V mutation, see Table 1 for letter codes of amino acids).

EC_{50} Concentration of a drug that produces a 50% effect, for example, in virus yield.

hemagglutinin Specific glycoprotein molecules on the surface of some viruses, which have the property of binding to the surface of the red blood cells of some animal species. Because there are multiple binding sites, one virus can bind to two red cells causing them to clump (agglutinate).

hepatotoxicity Liver toxicity.

interferon Any group of glycoproteins with antiviral activity.

lipoatrophy Redistribution/accumulation of body fat including central obesity, dorsocervical fat enlargement (buffalo hump), peripheral wasting, facial wasting, breast enlargement, and 'cushingoid appearance' observed in some patients receiving antiviral agents for HIV treatment.

maintenance therapy Drug treatment given for a long time to maintain its effect after the condition has been controlled or to prevent recurrence.

monotherapy Treatment with a single drug, contrasted with combination therapies with more than one drug at the same time.

mutations Changes to the base pair sequence of the genetic material of an organism.

nephrolithiasis The presence of kidney stones.

nephrotoxicity Kidney toxicity.

neuraminidase An enzyme, present on the surface of some viruses, which catalyzes the cleavage of a sugar derivative called neuraminic acid.

peptidomimetic A molecule having properties similar to those of a peptide or short protein.

pharmacokinetic Refers to the rates and efficiency of uptake, distribution, and disposition of a drug in the body.

phase III The final stage in testing of a new drug, after determination of its safety and effectiveness, in which it is tested on a broad range, and large population of patients for comparison to existing treatments and to test for rare complications.

placebo An agent used as a 'control' in tests of drugs. The placebo is an agent without the specific effects of the drug under test and is used to determine to what extent any observed effects of the drug are due to psychological effects or expectations. It is usually given to some patients while the test drug is given to others, but neither group knows which agent it is receiving (the so-called 'blind' design).

prodrug A drug that is given in a form that is inactive and must be metabolized in the body to the active form.

prophylaxis Prevention.

protease An enzyme that catalyzes the cleavage of proteins. In the case of HIV, a virus-specific protease is needed to cleave some of the virus coat proteins into their final, active form.

protease inhibitor A substance that inhibits the action of protease enzyme.

replication cycle The series of steps that a virus or cell goes through to multiply.

shingles Eruptive rash, usually in a girdle (L. cingulus; hence 'shingles') distribution on the trunk, resulting from infection with varicella zoster virus.

$T_{1/2}$ The time for reduction of some observed quantity, for example, the blood concentration of a drug, by 50%.

teratogenesis Production of fetal abnormalities by some agent.

therapeutic index The numerical ratio of the concentration needed to achieve a desired effect in 50% of the patients and the concentration that produces unacceptable toxicity in 50% of the patients.

thymidine kinase An enzyme that catalyzes the transfer of a phosphoryl group from a donor such as adenosine triphosphate to the sugar (deoxyribose) component of the thymidine molecule, a building block of DNA.

viremia The presence of virus in the bloodstream.

virion A complete virus, including the coat and nucleic acid core.

Abbreviations

ALT	alanine aminotransferase
CMV	cytomegalovirus
CNS	central nervous system disease
CoV	coronavirus
CPK	creatinine phosphokinase
CSF	cerebrospinal fluid
CYP	cytochrome P450
dATP	deoxyadenosine triphosphate
dGTP	deoxyguanosine triphosphate
EBV	Epstein–Barr virus
FDA	Food and Drug Administration
FEV1	reduced forced expiratory volume
HAART	highly active antiretroviral therapy
HBeAg	hepatitis B e antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HHV-6	human herpes virus-6
HHV-7	human herpes virus-7
HHV-8	human herpes virus-8
HIV	human immunodeficiency virus
HPMPC	(S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine
HPV	human papillomavirus
HSE	herpes simplex encephalitis
HSV	herpes simplex virus
IFNs	interferons
MNR	multinucleoside resistance
NA	neuraminidase
NAMs	nucleoside-analog-associated mutations
NK	natural killer
PEG	polyethylene glycol
Pgp	P-glycoprotein
RSV	respiratory syncytial virus
SARS	severe acute respiratory syndrome
SEM	skin, eye, or mouth
SPAG	small-particle aerosol generator
TAMs	thymidine analog resistance mutations
TK	thymidine kinases
UGT	UDP-glucuronosyl transferase
VZV	varicella zoster virus

Defining Statement

Antiviral agents are drugs approved by Food and Drug Administration (FDA) for the treatment or control of viral infections. They target stages in the viral life cycle. An ideal antiviral agent should be effective against both actively replicating and latent viruses; however, most of the available antiviral agents are effective against only replicating viruses.

Introduction

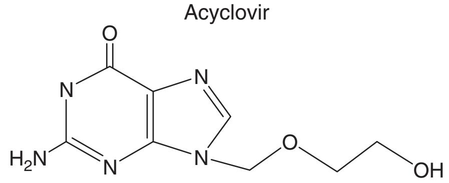
Antiviral agents are drugs approved by the Food and Drug Administration (FDA) for the treatment or control of viral infections. The development of antiviral agents is not trivial as viral replication is intricately linked with the host cell that any antiviral drug that interferes even to a lesser extent with host cell factors may be toxic to the host depending on the duration and dosage used. Available antiviral agents mainly target stages in the viral life cycle. The target stages in the viral life cycle are; viral attachment to host cell, uncoating, synthesis of viral mRNA, translation of mRNA, replication of viral RNA and DNA, maturation of new viral proteins, budding, release of newly synthesized virus, and free virus in body fluids. Antiviral agents used to treat viral diseases are currently limited, and at least half of the available agents are for the treatment of human immunodeficiency virus (HIV) infections. The others are used for the management of herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus (CMV), hepatitis B virus (HBV), hepatitis C virus (HCV), respiratory syncytial virus (RSV), human papillomavirus (HPV), and influenza virus-related diseases.

Viruses could stay in the cells as episomal form, or are incorporated into host chromosomal DNA without engaging in active viral replication (i.e., viral latency state). An ideal antiviral agent should be effective against both actively replicating and latent viruses; however, most of the available antiviral agents are effective against only replicating viruses. The goals for treating acute viral infections in immunocompetent patients are to reduce the severity of the illness and its complications and to decrease the rate of transmission of the virus. The therapeutic index, or ratio of efficacy to toxicity, must be extremely high in order for the therapy to be acceptable. For chronic viral infections, the goal is to prevent viral damage to visceral organs, and therefore efficacy becomes paramount. Antiviral agents can be used for prophylaxis, suppression, preemptive therapy, or treatment of overt disease. Two important factors that can limit the utility of antiviral drugs are toxicity and the development of resistance to the antiviral agent by the virus. In addition, host phenotypic behaviors toward antiviral drugs because of either genomic or epigenetic factors could limit the efficacy of an antiviral agent in an individual. This article summarizes the most relevant pharmacologic and clinical properties of the available antiviral agents.

Therapeutics for Herpesvirus Infections

There are eight members of the human herpesviridae family: HSV-1, HSV-2, VZV, Epstein–Barr virus (EBV), CMV, human herpes virus-6 (HHV-6), human herpes virus-7 (HHV-7), and human herpes virus-8 (HHV-8). The hallmark of the herpesviruses is their ability to establish latency within the neuronal ganglia of the nervous system or cells of the immune system and reactivate during periods of stress, trauma, or immune suppression.

Acyclovir



Chemistry, mechanism of action, and antiviral activity

Acyclovir [9-(2-hydroxyethoxymethyl) guanine] is a synthetic acyclic purine nucleoside analogue that lacks the 3′-hydroxyl group of nucleosides. Acyclovir is phosphorylated to the active triphosphate metabolite that inhibits viral DNA synthesis (**Figure 1**). Viral encoded thymidine kinases (TK), present in only herpesvirus-infected cells, catalyze the phophorylation to acyclovir monophosphate. Host cell TK or other kinases cannot phosphorylate acyclovir to its monophosphate metabolite efficiently. Acyclovir is highly selective for cells engaged in active viral replication and does not affect noninfected cells. The monophosphate is subsequently phosphorylated to the di-, and triphosphate by cellular kinases, resulting in acyclovir triphosphate concentrations much higher in HSV-infected than in uninfected cells. Acyclovir triphosphate inhibits viral DNA synthesis by competing with deoxyguanosine triphosphate (dGTP) as a substrate for viral DNA polymerase, as illustrated in **Figure 1**. Since acyclovir triphosphate lacks the 3′-hydroxyl group required for DNA chain elongation, the growing chain of DNA is terminated. In addition, the incorporated acyclovir can trap viral DNA polymerase and prevent it from initiating other viral DNA replication. The viral polymerase has a greater affinity for acyclovir triphosphate than cellular DNA polymerase, resulting in little incorporation of acyclovir into cellular DNA. *In vitro*, acyclovir is most active against HSV-1 (average $EC_{50} = 0.04\,\mu g\,ml^{-1}$), HSV-2 ($0.10\,\mu g\,ml^{-1}$), and VZV ($0.50\,\mu g\,ml^{-1}$). Varicella virus is much less susceptible to acyclovir than is HSV, and hence, higher doses of acyclovir are required in the treatment of VZV infections. EBV TK has poor efficiency to utilize acyclovir as substrate, therefore, higher acyclovir concentrations are required for EBV inhibition. CMV, which lacks a virus-specific TK, is relatively resistant.

The bioavailability of oral formulations of acyclovir is 15–30%. Peak concentrations of approximately 0.57 and $1.57\,\mu g\,ml^{-1}$ are attained after multidose oral administration of 200 or 800 mg of acyclovir, respectively. Higher plasma acyclovir levels are achieved with intravenous administration. The plasma half-life is 2–3 h in older children and adults with normal renal function and 2.5–5 h in neonates with normal creatinine clearance. The elimination

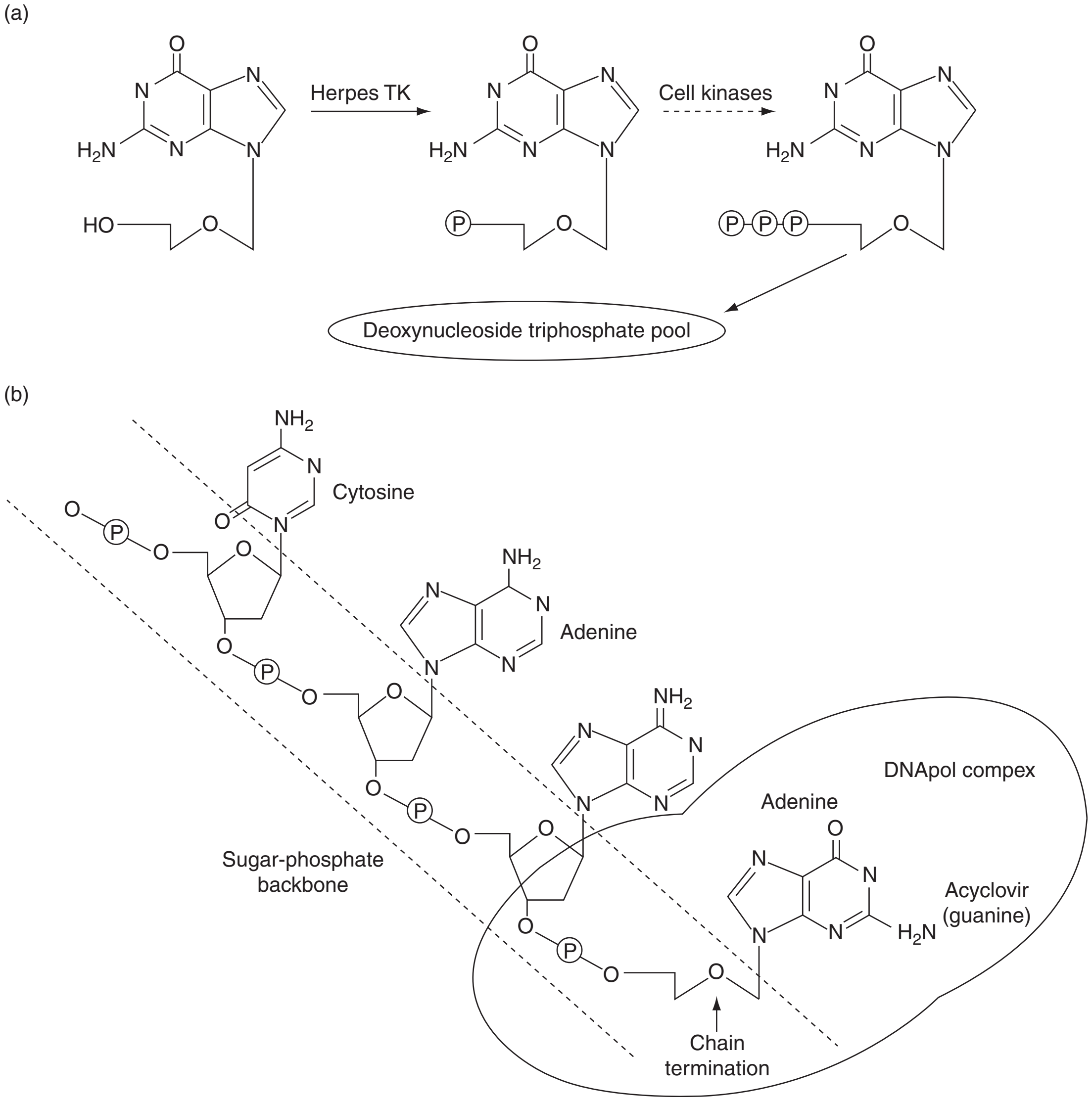


Figure 1 The mechanism of action of acyclovir: (a) activation; (b) inhibition of DNA synthesis and chain termination.

of acyclovir is prolonged in individuals with renal dysfunction, with a half-life of approximately 20 h in persons with end-stage renal disease. Acyclovir is minimally metabolized and approximately 85% is excreted unchanged in the urine via renal tubular secretion and glomerular filtration.

Clinical indications

For most of the clinical indications of acyclovir, valacyclovir and famciclovir are as effective, safe, and convenient alternatives. The clinical applications of valacyclovir and famciclovir are detailed in section 'Clinical indications' under 'Valacyclovir' and in section Clinical indications under 'Penciclovir and Famciclovir', respectively.

Genital herpes

Initial and recurrent episodes of genital HSV infection can be treated with acyclovir, and recurrent episodes can be suppressed with acyclovir. Topical acyclovir is not an effective treatment for genital HSV. Intravenous acyclovir (15 mg kg^{-1} day^{-1} in three divided doses for 5–7 days) is the most effective treatment for a first episode of genital herpes and results in a significant reduction in the median duration of viral shedding, pain, and time to complete healing (8 vs. 14 days) but is reserved for patients with systemic complications. Oral therapy (200 mg five times daily) is the standard treatment.

Recurrent genital herpes is less severe and resolves more rapidly than primary infection. Orally administered acyclovir (200 mg five times daily or 400 mg three times daily) for 7–10 days shortens the duration of signs and symptoms, virus shedding, and time to healing by 2, 7, and 4 days, respectively, when initiated within 24 h of onset of symptoms.

Oral acyclovir administration effectively suppresses recurrent genital herpes. Daily administration of acyclovir reduces the frequency of recurrences by up to 80%, and 25–30% of patients have no further recurrences while taking the drug.

Herpes labialis

Topical therapy for HSV-1 mouth or lip infections is of no clinical benefit. Orally administered acyclovir (200 or 400 mg five times daily for 5 days) reduces the time to loss of crust by approximately 1 day (7 vs. 8 days) but does not alter the duration of pain or time to complete healing.

Immunocompromised host

Immunocompromised individuals, such as those infected with HIV or transplant recipients, are afflicted with frequent and severe HSV infections. Clinical benefit from intravenous or oral acyclovir therapy is documented as evidenced by a significantly shorter duration of viral shedding and accelerated lesion healing. Oral acyclovir therapy in high doses in immunocompromised patients with herpes zoster is effective but valaciclovir is superior.

Herpes simplex encephalitis

HSV infection of the brain is the most common cause of sporadic fatal encephalitis in the United States. HSV-1 is predominantly the causative agent of herpes simplex encephalitis (HSE). Acyclovir at a dose of $10\,\mathrm{mg\,kg^{-1}}$ every 8 h ($30\,\mathrm{mg\,kg^{-1}\,day^{-1}}$) for 10–14 days is the therapy of choice and reduces mortality from 70 to 19%. Furthermore, 38% of acyclovir recipients returned to normal neurologic function.

Neonatal HSV infection

Neonatal HSV infection is divided into three clinical categories: skin, eye, or mouth (SEM) disease, central nervous system (CNS) disease, and disseminated (if there is evidence of visceral involvement) HSV disease. The recommended treatment for neonatal herpes infection is $20\,\mathrm{mg\,kg^{-1}}$ every 8 h of parenteral acyclovir with duration dictated by the extent of disease; 14 days for SEM disease, 21 days for CNS and disseminated disease. For babies with SEM disease, 98% of acyclovir recipients developed normally 2 years after infection. For babies surviving encephalitis and disseminated disease, 43 and 57% of acyclovir recipients, respectively, developed normally.

Varicella

Varicella or chicken pox, is a common, highly contagious illness caused by VZV. It is primarily a disease of early childhood with 90% of cases occurring in children 1–14 years of age. Chicken pox is generally self-limiting in young children and is manifested by fever, mild constitutional symptoms, and an itchy, vesicular rash. The disease is more severe in neonates, adults, and immunocompromised individuals. Oral acyclovir therapy when initiated within 24 h of the onset of the rash reduces the duration of fever, and the number of maximum lesions in immunocompetent children. At present, the clinical importance of acyclovir treatment in otherwise healthy children, in whom chicken pox is self-limiting and results in few complications, remains uncertain. Furthermore, the widely use of the varicella vaccine to protect against VZV will make the use of acyclovir for immunocompetent children with chickenpox obsolete.

Acyclovir therapy of chicken pox in immunocompromised host substantially reduces morbidity and mortality. Intravenous acyclovir treatment ($500\,\mathrm{mg\,m^{-2}}$ of body surface area or $10–12\,\mathrm{mg\,kg^{-1}}$ every 8 h for 7–10 days) improved the outcome, as evidenced by a reduction of VZV pneumonitis from 45 to <5%. Oral acyclovir therapy is not indicated for immunocompromised host with chicken pox. The bioavailability of valacyclovir makes it an attractive alternative.

Herpes zoster

Herpes zoster or singles is caused by the reactivation of VZV, which resides in a latent state in the sensory ganglia following primary varicella (chicken pox) infection. Acute herpes zoster is a painful, debilitating condition, especially in older adults. The risk of zoster-associated pain persisting after the healing of the rash correlates with increasing age. Intravenous acyclovir therapy of herpes zoster in the normal host produces some acceleration of the healing of the rash, and resolution of pain (both acute neuritis and zoster-associated pain). Oral acyclovir (800 mg five times a day) administration results in accelerated healing of the rash and reduction in the severity of acute neuritis. Oral acyclovir treatment of zoster ophthalmicus reduces the incidence of serious ocular complications such as keratitis and uveitis. Intravenous acyclovir therapy significantly reduces the frequency of cutaneous dissemination and visceral complications of herpes zoster in immunocompromised adults. Acyclovir is the standard therapy at a dose of $10\,\mathrm{mg\,kg^{-1}}$ (body weight) or $500\,\mathrm{mg\,m^{-2}}$ (body surface area) every 8 h for 7–10 days.

Resistance

The most common mechanism for conferring acyclovir resistance is mutations in the HSV genome resulting in a deficiency or alteration in viral TK activity. Occasionally, HSV strains are TK altered and maintain the ability to phosphorylate the natural substrate, thymidine, but selectively lose the ability to phosphorylate acyclovir. Mutation of the viral DNA polymerase gene resulting in failure to incorporate the acyclovir triphosphate in progeny DNA molecules is an alternate, but infrequent, mechanism that may result in HSV resistance to acyclovir.

Resistance to acyclovir is uncommon, with prevalence of 0.1–0.4% and 5–6% in immunocompetent and immunocompromised patients, respectively. Acyclovir-resistant isolates of VZV have been identified much less frequently than acyclovir-resistant HSV but have been recovered from marrow transplant recipients and AIDS patients. The acyclovir-resistant VZV isolates all had altered or absent viral TK function but remained susceptible to vidarabine and foscarnet, which do not require viral TK for their activity.

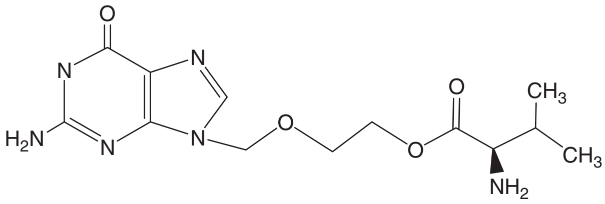
Adverse effect

Acyclovir therapy is associated with few adverse effects. The most common complaints associated with acyclovir therapy include nausea, diarrhea, and headache. Rapid infusions of intravenous acyclovir can result in reversible crystalline nephropathy. A few reports have linked intravenous acyclovir use with CNS disturbances, including agitation, hallucinations, disorientation, tremors, and myoclonus.

Data on outcomes from pregnant mothers exposed to acyclovir during pregnancy showed that the rate of birth defects did not exceed that expected in the general population and the pattern of defects did not differ from those in untreated women.

Valacyclovir

Valacyclovir



Chemistry, mechanism of action, and antiviral activity

Valacyclovir, is a prodrug of acyclovir (the L-valyl ester of acyclovir). Valacyclovir is rapidly metabolized into acyclovir and valine by the enzyme valacyclovir hydrolase (esterase) found in the brush border of the gastrointestinal tract, and the liver. Valacyclovir provides a high bioavailability of acyclovir, threefold to fivefold higher than that obtained with oral acyclovir, and is equivalent to plasma levels achieved with doses of intravenous acyclovir. The mechanism of action and antiviral activity spectrum of valacyclovir are similar to that as described for acyclovir.

Clinical indications

The antiviral spectrum of valacyclovir encompasses HSV-1, HSV-2, VZV, and CMV. It is effective for treatment of HSV-1 and HSV-2 infections in immunocompetent individuals; initial episode of genital herpes (1 g, twice daily for 10 days); episodic therapy for recurrent herpes labialis (2 g, twice a day for 1 day) and recurrent genital herpes (1 g or 500 mg, twice a day for 3–5 days); and suppression of recurrent genital herpes (1 g or 500 mg, once a day).

For immunocompromised patients, valacyclovir is effective for episodic therapy (1 g, twice a day for >5 days) and suppression of recurrent genital herpes (500 mg, twice a day, or 1 g, once a day). Valacyclovir (1 g, three times daily for 7–10 days) is superior to acyclovir for the reduction of pain associated with shingles.

Resistance

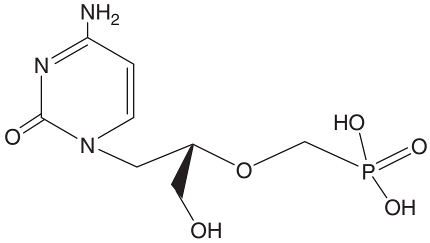
The mechanism of resistance to valacyclovir is similar to that of acyclovir.

Adverse effects

Valacyclovir has similar side effect profile as acyclovir; however, no crystalline nephropathy has been reported with its use.

Cidofovir

Cidofovir



Chemistry, mechanism of action, and antiviral activity

Cidofovir, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC), is an acyclic phosphonate nucleotide analogue of deoxycytidine monophosphate. Cidofovir has a single phosphate group attached therefore it does not require viral enzymes for conversion to the monophosphate, cellular kinases sequentially phosphorylate the monophosphate to its active triphosphate metabolite. The triphosphate metabolite then serves as a competitive inhibitor of DNA polymerase. The active form of the drug exhibits a 25- to 50-fold greater affinity for the viral DNA polymerase, compared with the cellular DNA polymerase, thereby selectively inhibiting viral replication. Owing to its unique phosphorylation requirements for activation, cidofovir usually maintains activity against acyclovir- and foscarnet-resistant HSV isolates, as well as ganciclovir- and foscarnet-resistant CMV mutants. Cidofovir is less potent than acyclovir *in vitro*; however, cidofovir persists in cells for prolonged periods, increasing drug activity. In addition, cidofovir produces active metabolites with long half-lives (17–48 h), permitting once weekly dosing. Cidofovir has *in vitro* activity against

VZV, EBV, HHV-6, HHV-8, HPV, polyomaviruses, orthopoxviruses, and adenovirus. Unfortunately, cidofovir concentrates in kidney cells 100 times greater than in other tissues and produces severe proximal convoluted tubule nephrotoxicity when administered systemically. Cidofovir has limited and variable oral bioavailability (2–26%), therefore, it is administered intravenously.

Clinical indication

Cidofovir is licensed for treatment of CMV retinitis and has been used to treat acyclovir-resistant HSV infection. The dosing regimen is 5 mg kg^{-1} per week during the first 2 weeks, then 5 mg kg^{-1} every other week, with sufficient hydration and coadministration of oral probenecid to prevent nephrotoxicity. There are anecdotal reports that dividing the 5 mg kg^{-1} weekly dose into three doses given alternate days in a week may reduce renal toxicity substantially.

Resistance

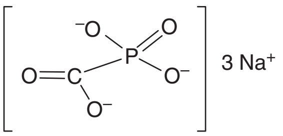
The development of resistance with clinical use is uncommon; however, mutations in CMV DNA polymerase can mediate altered susceptibility.

Adverse effects

Nephrotoxicity is the principal adverse event associated with systemic administration of cidofovir, occurs in 30–50% of recipients. Other reported side effects include neutropenia, fever, diarrhea, nausea, headache, rash, anterior uveitis, and ocular hypotonia.

Foscarnet

Foscarnet



Chemistry, mechanism of action, and antiviral activity

Foscarnet, is an inorganic pyrophosphate analogue of phosphonoacetic acid that inhibits all HHVs, including most ganciclovir-resistant CMV isolate and acyclovir-resistant HSV and VZV strains. It inhibits DNA polymerase by blocking the pyrophosphate-binding site and preventing cleavage of pyrophosphate from deoxynucleotide triphosphates. Unlike acyclovir, which requires activation by a virus-specific TK, foscarnet acts directly on the virus DNA polymerase. Thus, TK-deficient, acyclovir-resistant herpesviruses remain sensitive to foscarnet.

The oral bioavailability of foscarnet is about 20%. The cerebrospinal fluid (CSF) concentration of foscarnet is approximately two-thirds of the plasma level. Renal excretion is the primary route of clearance of foscarnet with >80% of the dose appearing in the urine. Bone sequestration also occurs, resulting in complex plasma elimination.

Clinical indications

The principal indications are CMV retinitis in AIDS patients, and mucocutaneous acyclovir-resistant (viral TK-deficient) or penciclovir-resistant HSV and VZV infections in immunocompromised patients.

Mucocutaneous HSV infections and those caused by VZV in immunocompromised host can be treated with foscarnet at dosages lower than that for the management of CMV retinitis.

Resistance

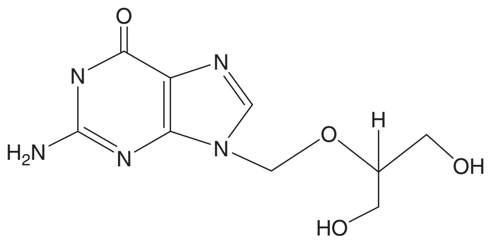
Isolates of HSV, CMV, and VZV resistant to foscarnet develop both in the laboratory and in the clinical setting. These isolates are all DNA polymerase mutants.

Adverse effects

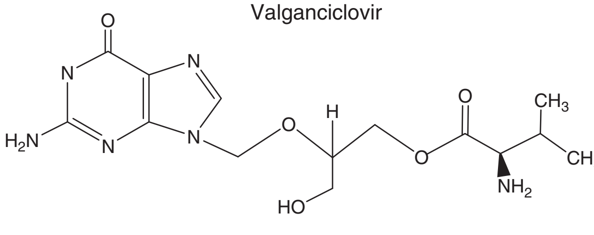
Foscarnet toxicity includes mainly nephrotoxicity (acute tubular necrosis and interstitial nephritis), metabolic and hematologic abnormalities, and CNS side effects. Patients may develop isolated or combined hypomagnesemia, hypocalcemia, hypokalemia, and hypophosphatemia. CNS side effects include headache, seizures, irritability, tremor, and hallucination. Other reported side effects include fever, rash, painful genital ulcerations, diarrhea, nausea, and vomiting.

Ganciclovir and Valganciclovir

Ganciclovir



Valganciclovir



Chemistry, mechanism of action, and antiviral activity

Ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine] is a nucleoside analogue that differs from acyclovir by having a hydroxymethyl group at the 3′ position of the acyclic side chain. It has 8–20 times greater *in vitro* activity against CMV, and as active as acyclovir against HSV-1 and HSV-2 and almost as active against VZV. Like acyclovir, the first step of phosphorylation to ganciclovir monophosphate in herpesvirus-infected cells depends on virus-encoded enzymes. In cells infected by HSV-1 or HSV-2, TK catalyzes the phosphorylation of ganciclovir to ganciclovir monophosphate. Because CMV lacks the gene for TK, the enzyme that catalyzes the initial phosphorylation of ganciclovir in CMV-infected cells is the phosphotransferase encoded by *UL97* gene. The final phosphorylation steps to the di- and triphosphate is by cellular kinases. Ganciclovir triphosphate serves as a competitive inhibitor of herpes viral DNA polymerase and inhibits the incorporation of guanosine triphosphate into viral DNA. Incorporation of ganciclovir triphosphate into the growing viral chain results in slowing and subsequent cessation of DNA chain elongation. Intracellular ganciclovir triphosphate concentrations are at least tenfold higher in CMV-infected cells than uninfected cells.

The oral bioavailability of ganciclovir is poor (5–7%). Concentrations of ganciclovir in biologic fluids, including aqueous humor and CSF, are less than plasma levels. The plasma elimination half-life is 2–4 h for individuals with normal renal function. The kidney is the major route of clearance of ganciclovir, and therefore, impaired renal function requires adjustment of dosage. The pharmacokinetics of ganciclovir in neonates is similar to that in adults.

Valganciclovir, L-valine ester of ganciclovir, serves as oral prodrug of ganciclovir. Valganciclovir is orally bioavailable (approximately 60%) and is rapidly converted to ganciclovir after absorption. Its mechanism of action and spectrum of activity are similar to that of ganciclovir. Oral valganciclovir can be given in doses that result in serum levels that approximate ganciclovir serum levels achieved with intravenous ganciclovir. Oral valganciclovir is convenient to use and may replace intravenous ganciclovir for initial and maintenance treatment.

Clinical indications

Ganciclovir is approved for treatment and chronic suppression of CMV retinitis in AIDS or other immunocompromised patients, and prophylaxis or preemptive treatment of CMV infection in high-risk transplant recipients. It is also effective for CMV syndromes, including CMV pneumonia, CMV colitis, and gastrointestinal infection in AIDS and transplant patients. In immunocompromised patients, therapy with ganciclovir requires an induction and maintenance phases. The induction dose is 10 mg kg^{-1} day^{-1} in two divided doses given for 14–21 days, and a maintenance dose of 5 mg kg^{-1} day^{-1} given once daily for 5–7 days per week. Oral valganciclovir dosage is 900 mg twice a day, and 900 mg once a day for induction and maintenance therapy, respectively.

Gancoclovir has been evaluated in the treatment of neonates congenitally infected with CMV. In a phase III randomized controlled trial, ganciclovir therapy (6 mg kg^{-1} per dose administered twice a day for 6 weeks) protected infants from hearing deterioration beyond one year of life. However, because of the potential toxicity of long-term ganciclovir therapy, additional studies are necessary before a recommendation can be made in the use of ganciclovir for congenital CMV infection.

Resistance

In immunocompromised patients receiving prolong therapy, the prevalence of resistance exceeds 8%. There are two mechanisms of resistance by CMV to ganciclovir: (1) The alteration of the CMV phosphonotransferase (coded by CMV *UL97* gene) reduces intracellular phosphorylation of ganciclovir, and (2) mutations in the CMV DNA polymerase (coded by CMV *UL54* gene). Resistance is associated with decreased sensitivity up to 20-fold. Occasionally, strains of HSV that are resistant to acyclovir because of TK deficiency are also much less sensitive to ganciclovir.

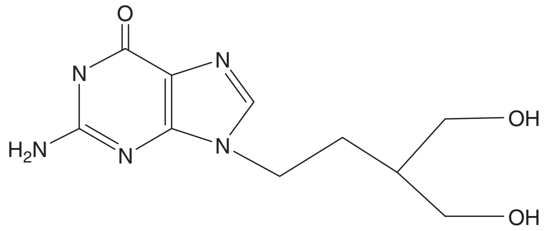
Adverse effects

The most important side effects of ganciclovir therapy are the development of neutropenia, and thrombocytopenia. Neutropenia occurs in approximately 24–38% of patients. The neutropenia is usually reversible with dosage adjustment of ganciclovir, or withholding of treatment. Thrombocytopenia occurs in 6–19% of patients.

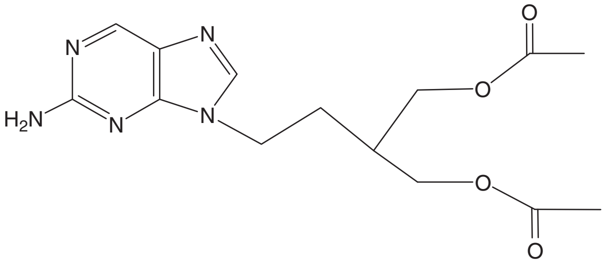
Ganciclovir has gonadal toxicity in animal models, most notably as a potent inhibitor of spermatogenesis. It causes an increased incidence of tumors in the preputial gland of male mice, a finding of unknown significance. As an agent affecting DNA synthesis, ganciclovir has carcinogenic potential.

Penciclovir and Famciclovir

Penciclovir



Famciclovir



Chemistry, mechanism of action, and antiviral activity

Penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)] a guanine nucleoside analogue is structurally similar to ganciclovir, differing only be the substitution of a methylene bridge for the ether oxygen in the acyclic ribose part of the molecule. Its metabolism and mechanism of action are similar to those of acyclovir, except that it is not an obligate DNA-chain terminator. The *in vitro* inhibitory effects of penciclovir on HSV-1, HSV-2, and VZV are similar to those of acyclovir. The oral bioavailability of penciclovir is poor (<5%). Famciclovir, a prodrug of penciclovir with improved bioavailability (approximately 77%), is the diacetyl ester of 6-deoxy penciclovir [9-(4-hydroxy-3-hydroxymethylbut-1-yl)-6-deoxyguanine]. It is well absorbed after oral administration and is rapidly metabolized to penciclovir by deacetylation in the gastrointestinal tract, and liver, after which it is oxidized by the liver at the position 6 of the purine ring. Penciclovir is phosphorylated more efficiently than acyclovir in HSV- and VZV-infected cells. Host cell kinases phosphorylate both penciclovir and acyclovir to a small but comparable extent. The preferential metabolism in HSV- and VZV-infected cells is the major determinant of its antiviral activity. Penciclovir triphosphate has, on average, a tenfold longer intracellular half-life than acyclovir triphosphate in HSV-1-, HSV-2-, and VZV-infected cells after drug removal. Because penciclovir is more stable, it has longer antiviral activity, allowing for less frequent dosing. Both compounds have good activity against HSV-1, HSV-2, and VZV. Penciclovir, like acyclovir, is relatively inactive against CMV and EBV. Penciclovir is active against hepatitis B.

Penciclovir is eliminated rapidly and almost unchanged by active tubular secretion and glomerular filtration by the kidneys. The elimination $T_{1/2}$ in healthy subjects is approximately 2 h.

Clinical indications

Famciclovir is available in an oral preparation for treatment of HSV-1, HSV-2, and VZV infections. It is used in the treatment of the following conditions: initial episodes of genital herpes (250 mg, three times a day for 10 days), episodic treatment of recurrent genital herpes (125 mg, twice a day for 5 days), suppression of recurrent genital herpes (250 mg, twice a day), and for shingles (500 mg, every 8 h for 7 days). For immunocompromised patients, famciclovir is efficacious for episodic treatment of recurrent genital herpes (500 mg, twice a day for 7 days). Compared with acyclovir, famciclovir is as effective, safe, and well tolerated in the treatment of HSV infections in HIV-infected individuals. Famciclovir is also at least as effective as acyclovir for ophthalmic zoster and for shingles and acute zoster pain in immunocompromised patients. Compared with valacyclovir, famciclovir is as effective, safe, and convenient in the treatment of zoster.

Penciclovir is available as a 1% cream for topical therapy of mucocutaneous HSV infections, particularly recurrent herpes labialis (cold sores). Topical penciclovir 1% is approved for episodic therapy of herpes labialis and applied every 2 h during waking hours for 4 days. It accelerates lesion healing and resolution of pain by about 1 day. It is available over-the-counter in many countries.

Resistance

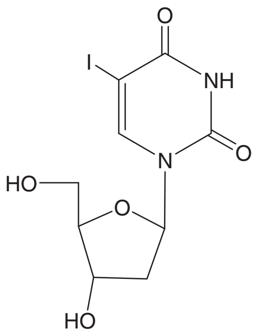
HSV and VZV isolates resistant to penciclovir have been identified in the laboratory. Resistance is attributed to alterations or deficiencies of TK and DNA polymerase.

Adverse effects

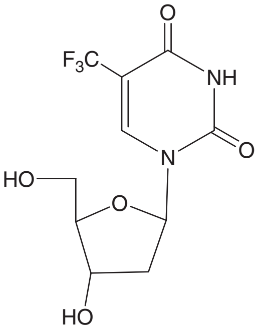
Therapy with oral famciclovir is well tolerated, being associated only with headache, diarrhea, and nausea. Preclinical studies of famciclovir indicated that chronic administration was tumorigenic (murine mammary tumors) and causes testicular toxicity in other rodents.

Idoxuridine and Trifluorothymidine

Idoxuridine



Trifluorothymidine



Chemistry, mechanism of action, and antiviral activity

Idoxuridine (5-iodo-2′-deoxyuridine), and trifluorothymidine (5-trifluoromethyl-2′-deoxyuridine) are thymidine analogue. When administered systemically, these nucleosides are phosphorylated by both viral and cellular kinases to active triphosphate derivatives, which inhibit both viral and cellular DNA synthesis. Parenteral administration results in potent antiviral activity but also sufficient host cytotoxicity to prevent the systemic use of these drugs. The toxicity of these compounds is not significant when applied topically to the eye in the treatment of HSV keratitis. Both idoxuridine and trifluorothymidine are effective and licensed for treatment of HSV keratitis. Topically applied idoxuridine or trifluorothymidine will penetrate cells of the cornea. Low levels of drugs can be detected in the aqueous humor.

Clinical indications

Trifluorothymidine is the most efficacious of these compounds, and the treatment of choice for HSV keratitis (1 drop of 1% ophthalmic solution instilled in each eye, up to nine times a day). Idoxuridine was the first antiviral compound to receive FDA approval in 1963 for treatment of HSV keratitis.

Resistance

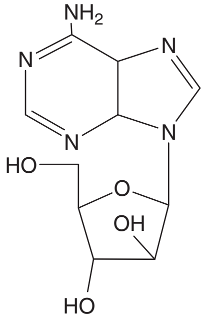
Trifluorothymidine-resistant HSV strains with altered TK substrate specificity have been selected for *in vitro*. However, clinical significant resistance has not been established.

Adverse effects

The ophthalmic preparation of idoxuridine and trifluorothymidine causes local discomfort, irritation, photophobia, edema of the eyelids, and less commonly, hypersensitivity reactions as well as superficial punctate or epithelial keratopathy.

Vidarabine

Vidarabine



Chemistry, mechanism of action, and antiviral activity

Vidarabine (vira-A, adenine arabinoside, and 9-D-arabinofuranosyl adenine) is active against HSV, VZV, and CMV. Vidarabine is a purine nucleoside analogue that is phosphorylated intracellularly to its mono-, di-, and triphosphate derivatives. Thus, unlike acyclovir, conversion of vidarabine to its active intracellular derivative does not require viral enzymes at any of the phosphorylation steps. The triphosphate derivative competitively inhibits DNA dependent DNA polymerases of some DNA viruses approximately 40 times more than those of host cells. In addition, vira-A is incorporated into terminal positions of both cellular and viral DNA, thus inhibiting elongation. Viral DNA synthesis is blocked at lower doses of drug than is host cell DNA synthesis, resulting in a relatively selective antiviral effect. However, large doses of vira-A are cytotoxic to dividing host cells.

The use of vidarabine was replaced by acyclovir because of poor solubility and toxicity. It is no longer available as an intravenous formulation. However, vidarabine should be recognized historically as the first antiviral agent licensed in 1977 for systemic treatment.

Clinical indications

Although trifluorothymidine is the antiviral agent of choice for the topical treatment of HSV keratitis, in patients in whom trifluorothymidine cannot be used vidarabine is a suitable alternative. Topical vidarabine is superior to idoxuridine in the treatment of HSV ocular infections.

Resistance

Resistance to vidarabine is conferred by mutations in the viral DNA polymerase gene. The degree of maximal resistance to vidarabine is fourfold, much lower than the 100-fold resistance to acyclovir with similar DNA polymerase resistant mutations. Acyclovir-resistant clinical HSV isolates are always susceptible *in vitro* to vidarabine.

Adverse effects

Ocular toxicity consists of occasional hyperemia and increased tearing, both of low incidence.

Fomivirsen

Chemistry, mechanism of action, and antiviral activity

Fomivirsen is a 21-nucleotide phosphorothioate oligonucleotide that inhibits CMV replication through an antisense mechanism. Its oligonucleotide sequence ($5'$-GCG TTT GCT CTT CTT CTT GCG-$3'$) is complementary to a sequence in mRNA transcripts of the major immediate early region 2 (IE2) of CMV, which encodes for several proteins responsible for the viral gene expression that are essential for the production of infectious viral particles. Binding of fomivirsen to the target mRNA results in inhibition of IE2 protein synthesis, with subsequent inhibition of viral replication. *In vitro*, fomivirsen inhibits CMV replication in a dose-dependent manner, with a mean IC_{50} of between 0.03 and $0.2\ \mu mol\ l^{-1}$. Pharmacokinetic assessment of fomivirsen in humans after intraocular administration is limited. In a rabbit model, intraocular administration revealed first-order kinetics with half-life of 62 h. Fomivirsen is cleared from the vitreous in rabbits during the course of 7–10 days by a combination of tissue distribution and metabolism. No systemic absorption has been observed after intravitreal administration in humans.

Clinical indications

Fomivirsen is indicated for use in HIV patients with CMV retinitis who are intolerant of or have contraindication to other treatment for CMV retinitis or in whom the disease is recalcitrant to ganciclovir or cidofovir treatment. It has activity against cidofovir- and ganciclovir-resistant strains of CMV.

Resistance

CMV strains with tenfold decreased susceptibility have been selected *in vitro*. However, no resistant clinical isolates have been reported.

Adverse effects

Adverse events of fomivirsen are uveitis, including iritis and vitritis, occurring in approximately 25% of patients. These reactions are usually transient or reversible with topical corticosteroids treatment.

New Prospects for Therapy of Herpesvirus Infections

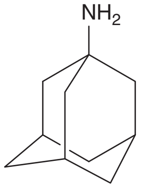
While several classes of compounds are being investigated for the treatment of herpesvirus infections. Some of the compounds that have been the focus of drug discovery in the last decade have been targets of viral encoded enzymes, including inhibitors of ribonucleotide reductase, TK, protease, and DNA polymerase.

Recently, a new compound class of helicase–primase inhibitors, for example, BAY 57-1293, with preclinical pharmacological profile that outperforms the current standard HSV treatment represented by acyclovir, valacyclovir and famciclovir in standard animal models with respect to all parameters of efficacy, has been discovered. This class of compounds bind to two viral targets simultaneously, namely the helicase and primase subunit of the helicase–primase enzyme complex. The helicase–primase complex is essential for the HSV DNA replication process. These compounds have no demonstrable activity against either VZV or CMV.

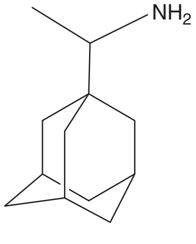
Therapeutics for Respiratory Virus Infections

Amantadine and Rimantadine

Amantadine



Rimantadine



Chemistry, mechanism of action, and antiviral activity

Amantadine (1-adamantanamine hydrochloride) and rimantadine (α-methyl-1-adamantanemethylamine hydrochloride) are symmetric tricyclic amines with narrow spectrum of activity, being useful only against influenza A infections. Rimantadine is fourfold to tenfold more active than amantadine. The mechanism of action of these drugs relates to the influenza A virus M2 protein, an integral transmembrane protein that functions as an ion channel for this virus and is activated by pH. The drop in pH accompanying the hydrogen flux facilitates the dissociation of the M2 protein from the ribonucleoprotein complexes so that the ribonucleoprotein can enter the cell nucleus and initiate replication. By interfering with the function of the M2 protein, amantadine and rimantadine inhibit the acid-mediated dissociation of the matrix protein from the ribonuclear protein complex within endosomes. This event occurs early in the viral replication cycle. The consequences of these drugs are the potentiation of acidic pH-induced conformational changes in the viral hemagglutinin during its intracellular transport.

Absorption of rimantadine is slower compared with that of amantadine. Amantadine is excreted unchanged in the urine by glomerular filtration and, likely, tubular secretion. The plasma elimination $T_{1/2}$ is approximately 12–18 h in individuals with normal renal function. However, the elimination $T_{1/2}$ increases in the elderly with impaired creatinine clearance. Rimantadine is extensively metabolized following oral administration, with an elimination $T_{1/2}$ of 24–36 h. Approximately 15% of the dose is excreted unchanged in the urine.

Clinical indications

Amantadine and rimantadine are licensed both for the chemoprophylaxis and treatment of influenza A infections. Prophylaxis with either drug prevents approximately 50–60% of infections and 70–90% of clinical illnesses caused by type A influenza virus. This degree of prophylactic effectiveness approximates that of inactivated influenza vaccine. Because of a lower incidence of side effects associated with rimantadine, it is used preferentially. Rimantadine can be given to any unimmunized member of the general population who wishes to avoid influenza A, but prophylaxis is especially recommended for control of presumed influenza outbreaks in institutions housing high-risk persons. High-risk individuals include adults and children with chronic disorders of the cardiovascular or pulmonary systems. Prophylaxis also is indicated if the vaccine may be ineffective because the epidemic strain differs substantially from the vaccine strain of influenza A, and for the 2 weeks after vaccination if influenza A already is active in the community.

Amantadine and rimantadine also have been shown to be effective in treatment of influenza A infections in adults and children if treatment is initiated within 48 h of the onset of symptoms. Drug therapy results in reduction in the duration of viral excretion, fever, and other systemic complaints, as well as earlier resumption of normal activities, in comparison with placebo. On average, the duration of illness is shortened by about 1 day. Amantadine and rimantadine are given orally at 200, and 300 mg day^{-1}, respectively.

Resistance

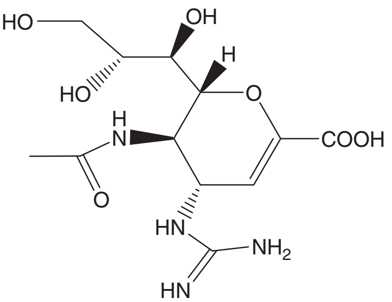
Resistance to amantadine and rimantadine results from point mutations in the RNA sequence encoding for the M2 protein transmembrane domain. Resistance typically appears in the treated subjects within 2–3 days of initiating therapy. About 25–35% of treated patients shed resistant strains by the 5th day of treatment. The clinical significance of isolating resistant strains from the treated subject is not clear; infection and illness in immunocompetent people infected with a drug-resistant virus are similar to those in patients infected with drug-sensitive virus.

Adverse effects

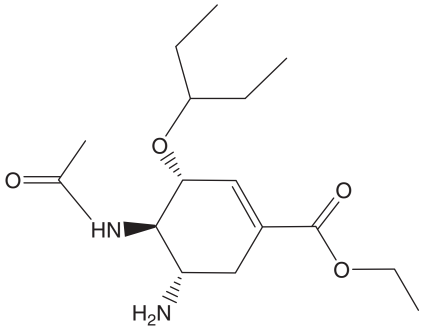
Although the spectrum of adverse events associated with amantadine and rimantadine are qualitatively similar, they are less frequent and less severe with rimantadine. Amantadine is reported to cause side effects in 5–10% of healthy young adults taking the standard adult dose of 200 mg day^{-1}. These side effects are usually mild and cease soon after amantadine is discontinued, although they often disappear with continued use of the drug. CNS side effects, which occur in 5–33% of patients, are most common and include difficulty in thinking, confusion, lightheadedness, hallucinations, anxiety, and insomnia. More severe adverse effects (e.g., mental depression and psychosis) are usually associated with doses exceeding 200 mg daily. About 5% of patients complain of nausea, vomiting, or anorexia. CNS adverse effects associated with rimantadine administration are significantly less; however, rimantadine has been associated with exacerbations of underlying seizure disorders.

Zanamivir and Oseltamivir

Zanamivir



Oseltamivir



Chemistry, mechanism of action, and antiviral activity

Zanamivir (4-guanidino-2,3-dideoxy-2,3-didehydro-*N*-acetylneuraminic acid), and oseltamivir [ethyl ester of (3*R*,4*R*,5*S*)-4-scetamido-5-amino-3-(1-ethylpropoxyl)-1-cyclohexane-1-carboxylic acid] are sialic acid analogue that competitively inhibit influenza virus neuraminidase (NA). Influenza virus NA is located on the surface of the virus and is responsible for cleaving terminal sialic acid residues, which are essential for the release of the virus from infected cells, viral aggregation, and spread within the respiratory tract. Influenza NA also decreases viral inactivation by respiratory mucous. The lipophilic side chain of zanamivir and oseltamivir binds to the influenza virus NA, blocking its ability to cleave sialic acid residues. Zanamivir and oseltamivir are effective against both influenza A and influenza B.

Zanamivir has poor oral bioavailability and therefore it is administered by oral inhalation. More than 75% of an orally inhaled dose of zanamivir is deposited in the oropharynx, approximately 13% of this is distributed to the airways and lungs. Local respiratory mucosal concentrations of zanamivir exceeds 1000 ng ml^{-1} in sputum for 6 h after inhalation (i.e., over and above the concentration required to inhibit influenza A and B viruses). Approximately 10% of inhaled dose is absorbed systemically; peak serum concentrations range from 17 to 142 ng ml^{-1} within 2 h of administration of a 10 mg dose. The plasma $T_{1/2}$ is between 2.5 and 5 h. Systemically absorbed zanamivir is excreted unchanged in the urine. Although serum concentrations of zanamivir increase with decreasing creatinine clearance, no adjustment in dosing is necessary for renal insufficiency because of the limited amount of systemically absorbed drug.

Oseltamivir is an ethyl ester prodrug that, following hydrolysis by hepatic esterases, is converted to the active compound, oseltamivir carboxylate. Approximately 75% of orally administered oseltamivir reaches the systemic circulation in the form of oseltamivir carboxylate. Oseltamivir carboxylate is eliminated unchanged by renal excretion through glomerular filtration and tubular secretion. The elimination $T_{1/2}$ of oseltamivir carboxylate is 6–10 h. Serum concentrations of the drug increase in the presence of declining renal function, and dose adjustment is recommended in patients with renal insufficiency.

Clinical indications

Zanamivir and oseltamivir are used for treatment and prevention of influenza A and B infections. Treatment of otherwise healthy adults and children with zanamivir and oseltamivir reduces the duration of symptoms by 0.4 and 1 days, and provides 29–43% relative reduction in the odds of complications when given within 48 h of onset of symptoms. These drugs also significantly diminish viral replication in respiratory secretions. Zanamivir is available as dry powder for inhalation using a breath-activated Diskhaler delivery system. The recommended dose of zanamivir in patients >7 years is 10 mg twice daily for 5 days, while oseltamivir is given at 75 mg twice a day for 5 days.

Inhaled zanamivir, 10 mg once daily given for 4 weeks as seasonal prophylaxis, reduces the likelihood of laboratory confirmed influenza (with or without symptoms) by 34%, influenza disease by 67%, and influenza disease with fever by 84%. Oseltamivir administered for 6 weeks during the peak of influenza season significantly reduces the risk of contracting influenza. The protective efficacy of oseltamivir in preventing culture-proven influenza is about 90%.

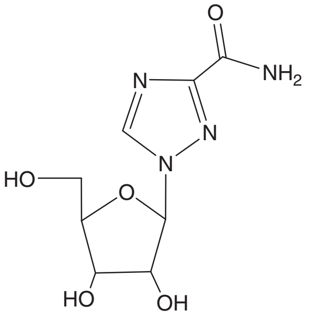
Resistance

Viruses resistant to zanamivir and oseltamivir have been generated after *in vitro* passage in cell culture. Clinical influenza virus isolates with reduced susceptibility to both NA inhibitors have been reported. There are two mechanisms of resistance: mutations in the hemagglutin receptor-binding site, and mutations in the conserved portions of the NA enzyme active site. In general, resistant viruses with mutations in the NA enzyme are thought to have decreased infectivity and fitness and therefore less likely to be transmitted.

Adverse effects

Both NA inhibitors are generally well tolerated. Adverse events following administration of oseltamivir have primarily been gastrointestinal with nausea and vomiting occurring in some patients. Inhalation of zanamivir has resulted in bronchospasm and reduced forced expiratory volume (FEV1). Zanamivir should be used with caution in individuals with reactive airway diseases or chronic obstructive pulmonary diseases.

Ribavirin



Ribavirin

Chemistry, mechanism of action, and antiviral activity

Ribavirin (α-methyl-1-adamantane methylamine hydrochloride) has antiviral activity against a variety of RNA and DNA viruses. Ribavirin is a nucleoside analogue whose mechanisms of action are poorly understood and probably not the same for all viruses; however, its ability to alter nucleotide pools and the packaging of mRNA appears important. This process is not virus specific, but there is a certain selectivity, in that infected cells produce more mRNA than noninfected cells. A major action is the inhibition by ribavirin-5′-monophosphate of inosine monophosphate dehydrogenase, an enzyme essential for DNA synthesis. This inhibition may have direct effects on the intracellular level of GMP and other nucleotide levels may be altered. The 5′-triphosphate of ribavirin inhibits the formation of the 5′-guanylation capping on the mRNA of vaccinia and Venezuelan equine encephalitis viruses. In addition, the triphosphate is a potent inhibitor of viral mRNA (guanine-7) methyltransferase of vaccinia virus. The capacity of viral mRNA to support protein synthesis is markedly reduced by ribavirin. Ribavirin may inhibit influenza A RNA-dependent RNA polymerase.

Ribavirin can be administered orally (bioavailability of approximately 40–45%) or intravenously. Aerosol administration has become standard for the treatment of RSV infections in children. Oral doses of 600 and 1200 mg result in peak plasma concentrations of 1.3 and 2.5 $\mu g\,ml^{-1}$, respectively. Intravenous dosages of 500 and 1000 mg result in 17 and 24 $\mu g\,ml^{-1}$ plasma concentrations, respectively. Aerosol administration of ribavirin results in plasma levels that are a function of the duration of exposure. Although respiratory secretions will contain milligram quantities of drug, only microgram quantities (0.5–3.5 $\mu g\,ml^{-1}$) can be detected in the plasma.

The kidney is the major route of clearance of drug, accounting for approximately 40%. Hepatic metabolism also contributes to the clearance of ribavirin. Notably, ribavirin triphosphate concentrates in erythrocytes and persists for a month or longer. Likely, the persistence of ribavirin in erythrocytes contributes to its hematopoietic toxicity.

Clinical indications

Respiratory syncytial virus

Ribavirin is licensed for the treatment of carefully selected, hospitalized infants and young children with severe lower respiratory tract infections caused by RSV. Use of aerosolized ribavirin in adults and children with RSV infections reduced the severity of illness and virus shedding. However, placebo controlled trials have failed to demonstrate a consistent decrease in need for mechanical ventilation, duration of stay in intensive care unit, or duration of hospitalization among ribavirin recipients. The use of ribavirin for the treatment of RSV infections is controversial and remains discretionary. The most common adverse events following aerosol administration of ribavirin include bronchospasm and malfunction of ventilator delivery systems. The usual dosage of aerosolized ribavirin is 20 $mg\,ml^{-1}$ of drug instilled in a small-particle aerosol generator (SPAG) and administered for 12–22 h day^{-1} for 3–6 days. To avoid potential exposure of health care workers to ribavirin, special containment delivery system in an isolation room with negative pressure is used.

Hepatitis C

Oral ribavirin in combination with interferon-α (IFN-α) is recommended for hepatitis C infection.

Lassa fever and hemorrhagic fever

Systemic ribavirin has demonstrated efficacy in the management of life-threatening infections caused by Lassa fever and hemorrhagic fever with renal syndrome. Oral ribavirin is recommended for prophylaxis against Lassa fever in exposed contacts.

Resistance

Treatment failures with ribavirin occur in some patients; however, resistance to ribavirin has not been identified as a clinical problem.

Adverse effects

Adverse effects attributable to aerosol therapy with ribavirin of infants with RSV include bronchospasm, pneumothorax in ventilated patients, apnea, cardiac arrest, hypotension, and concomitant digitalis toxicity. Pulmonary function test changes after ribavirin therapy in adults with chronic obstructive pulmonary disease have been noted. Reticulocytosis, rash, and conjunctivitis have been associated with the use of ribavirin aerosol. When given orally or intravenously, transient elevations of serum bilirubin and the occurrence of mild anemia have been reported. Ribavirin has been found to be teratogenic and mutagenic in preclinical testing. Its use is contraindicated in women who are or may become pregnant during exposure to the drug. Concern has been expressed about the risk to persons in the room of infants being treated with ribavirin aerosol, particularly females of childbearing age. Although this risk seems to be minimal with limited exposure, awareness and caution are warranted and, therefore, the establishment of stringent precautions for administration of ribavirin.

New Prospects for Therapy of Respiratory Viruses

While influenza pandemics have long posed a threat to humankind, a threat realized to varying extents in 1918, 1957, and 1968, particular concern has mounted of late due to continued sporadic human cases of H5N1 avian influenza in Southeast Asia, Eastern Europe, and Africa. Amantadine and rimantadine are not recommended for seasonal or avian influenza because circulating influenza A viruses as well as the H5N1 strains affecting humans in Southeast Asia are resistant to these medications. Zanamivir and oseltamivir are the only available options. To expand the antiviral drug arsenal against influenza, researchers have been testing a number of investigational agents, including peramivir and T-705. Although peramivir is a NA inhibitor administered intramuscularly. Preclinical studies in mice and ferrets revealed that the drug could protect 80% or more of animals exposed to pathogenic H5N1 influenza virus compared with 36% of untreated animals. T-705 is a viral RNA polymerase inhibitor in the preclinical testing stage.

The outbreak in 2003 of a novel coronavirus infection, which causes severe acute respiratory syndrome (SARS), underscores the need for antiviral drugs for the control of future SARS outbreaks. New insights into the field of SARS pathogenesis and SARS-coronavirus (CoV) genome structure have revealed novel potential therapeutic targets for antiviral therapy.

Therapeutics for Hepatitis

Interferons

Chemistry, mechanism of action, and antiviral activity

IFNs are glycoprotein cytokines (intercellular messengers) with a complex array of immunomodulating, antineoplastic, and antiviral properties. IFNs are currently classified as α, β, or γ, the natural sources of which, in general, are leukocytes, fibroblasts, and lymphocytes, respectively. Each type of IFN can be produced through recombinant DNA technology. The binding of IFN to the intact cell membrane is the first step in establishing an antiviral effect. IFN binds to the cellular receptors and activates secondary messengers to initiate production of multiple proteins, which are pivotal for the defense of the cell against viruses. The mechanism of action is rather complex. The antiviral effects of IFN include degradation of viral mRNA, inhibition of viral protein synthesis, and prevention of the viral infection of cells. The immunomodulating effects of IFN include enhancement of antigen presentation by HLA I and II to the immune system, activation of natural killer (NK) cells and other immune cells, and increased cytokine production. IFNs are active against a wide spectrum of viruses with RNA viruses being more sensitive than DNA viruses.

IFNs are not orally bioavailable and are administered intramuscularly or subcutaneously (including into a lesion). There appears to be some variability in absorption between each of the three classes of IFN and, importantly, resultant plasma levels. Absorption of IFN-α is more than 80% complete after intramuscular or subcutaneous injection. Plasma levels are dose dependent, peaking 4–8 h after administration and returning to baseline between 18 and 36 h. However, the antiviral activity peaks at 24 h and then slowly decreases to baseline over about 6 days. IFN is eliminated by inactivation in various body fluids and metabolism in a number of organs. Negligible amounts are excreted in the urine unchanged.

IFN-α molecule covalently bonded to polyethylene glycol (PEG) improves the pharmacokinetic profile of IFN markedly. The pegylated forms of IFN-α (Peg-IFN-α) offer a more convenient once weekly instead of daily administration, are licensed for the treatment of hepatitis B and C.

Clinical indications

Hepatitis B

The major goals of therapy for hepatitis B are to prevent progression of the disease to cirrhosis, end stage liver disease or hepatocellular carcinoma. Three generally accepted indications for treatment are: (1) positive test for HBV DNA, (2) positive hepatitis B e antigen (HBeAg), and (3) alanine aminotransferase (ALT) level greater than two times normal. Treatment end points differ in HBeAg positive, and HBeAg negative disease. However, suppression of HBV replication to levels less than 1×10^4 copies per ml (2000 IU ml^{-1}) is regarded as a surrogate of treatment success with a resultant improvement in serum ALT and hepatic necroinflammatory disease.

Hepatitis B DNA polymerase level, a marker of replication, is reduced with standard IFN therapy. Clearance of serum HBeAg and HBV DNA polymerase occurs with treatment (30–40%). The use of pegylated forms of IFN has become common with the convenience of weekly dosing. Genotype and other baseline factors affect the response to PEG-IFN-α2a in HBeAg-positive chronic hepatitis B. Patients with genotypes A and B have a better response in comparison with genotypes C and D patients.

Hepatitis C

The aim of therapy for chronic HCV infection is to decrease and ultimately prevent progressive liver damage leading to cirrhosis, liver failure, or hepatocellular carcinoma. Therapy for chronic HCV infection is indicated in patients with detectable HCV RNA viral load and persistently elevated ALT. Findings of cirrhosis, fibrosis, or even moderate inflammation on liver biopsy support the choice of therapeutic intervention; however, biopsy is not mandatory prior to treatment initiation. Standard IFN, either as monotherapy or in combination with ribavirin, has been used extensively for HCV infections. Combination therapy for 40 weeks resulted in sustained responses in more than 60% of patients. The standard treatment of HCV infection is either PEG-IFN-α2a or PEG-IFN-α2b given alone or in combination with ribavirin. Genotypes 1 and 4 infections are associated with lower sustained virologic response than other HCV genotypes.

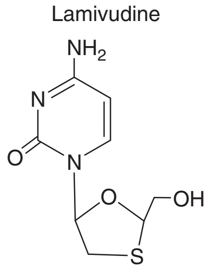
Resistance

Resistance to administered IFN has not been documented although neutralizing antibodies to recombinant IFNs have been reported. The clinical importance of this latter observation is unknown.

Adverse effects

Side effects are frequent with IFN (both standard and pegylated) administration and are usually dose limiting. Influenza-like symptoms (i.e., fever, chills, headache, and malaise) commonly occur, but these symptoms usually become less severe with repeated treatments. Leukopenia is the most common hematologic abnormality, occurring in up to 26% of treated patients. Leukopenia is usually modest, not clinically relevant, and reversible upon discontinuation of therapy. Increased ALT levels may also occur as well as nausea, vomiting, and diarrhea. At higher doses of IFN, neurotoxicity is encountered, as manifested by personality changes, confusion, attention deficits, disorientation, and paranoid ideation.

Lamivudine



Chemistry, mechanism of action, and antiviral activity

Lamivudine is the (–) enantiomer of a cytidine analogue with sulfur substituted for the 3′ carbon atom in the furanose ring [(–) 2′,3′-dideoxy, 3′-thiacytidine]. It has significant activity *in vitro* against both HIV-1 and HIV-2 as well as HBV. Lamivudine is phosphorylated to the triphosphate metabolite by cellular kinases. The triphosphate derivative is a competitive inhibitor of the viral reverse transcriptase.

The oral bioavailability in adults is >80% for doses between 0.25 and 8.0 mg kg^{-1}. Peak serum concentrations of 1.5 μg ml^{-1} are achieved in 1–1.5 h and the plasma $T_{1/2}$ is approximately 2–4 h. Lamivudine is eliminated by the kidneys unchanged by both glomerular filtration and tubular excretion, and dosages should be adapted to creatinine clearance.

Clinical indications

Lamivudine is effective as monotherapy for the treatment of chronic HBV infection and in combination with other antiretroviral drugs for treatment of HIV infection. It is the first line drug for the treatment of HBeAg and anti-HBe positive disease. Elevated serum ALT levels have been shown to predict a higher likelihood of HBeAg loss in patients with chronic hepatitis B treated with lamivudine. Lamivudine is administered orally at 100 mg day^{-1} in the treatment of HBV infections, though the ideal dose could be higher.

Resistance

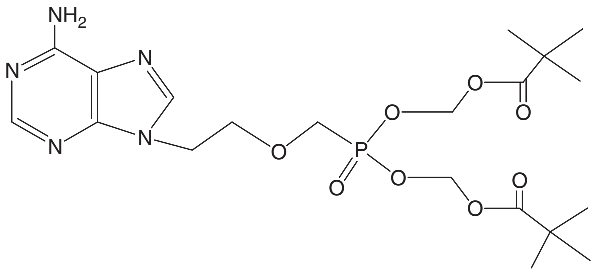
Resistance to lamivudine monotherapy develops within 6 months of therapy. The incidence of lamivudine resistance is 15–20% per year, with 70% patients becoming resistant after 5 years of treatment. It will be curious to know if lamivudine at higher doses will affect the incidence of resistance. Lamivudine resistance to HBV is conferred through HBV strains with mutations in the viral polymerase, within the catalytic domain (C domain), which includes the YMDD motif (e.g., M204V or M204I), and within the B domain (e.g., L180M or V173L). These mutants have a reduced replication capacity compared with the wild type HBV virus. Lamivudine resistance is managed by sequential treatment with either adefovir or entecavir. However, the advantage of sequential treatment compared to *de novo* combination therapy is questionable.

Adverse effects

Lamivudine has an extremely favorable toxicity profile. This may be partly because lamivudine does not affect mitochondrial DNA synthesis and its poor inhibition of human DNA polymerases. At the highest doses of 20 mg kg^{-1} day^{-1}, neutropenia is encountered but at a low frequency.

Adefovir Dipivoxil

Adefovir dipivoxil



Chemistry, mechanism of action, and antiviral activity

Adefovir dipivoxil, bis(pivaloyloxymethyl)ester of 9-(2-phosphonylmethoxyethyl) adenine, is an orally bioavailable prodrug of adefovir, a phosphonate acyclic nucleotide analogue of adenosine monophosphate. Adefovir is monophosphorylated and is not dependent on initial phosphorylation by viral nucleoside kinases to exert its antiviral effect. The phosphorylation to the di- and triphosphate metabolites is by cellular kinases. The triphosphate competes with endogenous deoxyadenosine triphosphate (dATP) in incorporation to the nascent viral DNA resulting in premature termination of viral DNA synthesis due to the lack of a 3′ hydroxyl group. It has activity against HIV, hepadnaviruses and herpesviruses. The bioavailability of adefovir dipivoxil in humans is about 40%. It has a long intracellular half-life of 18 h allowing for a once-daily dose. Clearance of adefovir is by renal excretion. Its pharmacokinetics is substantially altered in subjects with moderate and severe renal impairment.

Clinical indications

The efficacy of adefovir has been assessed in patients with HBeAg positive and negative disease and other settings in the spectrum of chronic hepatitis B infection. At the recommended dose of 10 mg once a day, adefovir resulted in significant improvement when compared with placebo: improvement in liver histology (53% vs. 25%), reduction in HBV DNA (3.52 vs. 0.55 log copies ml^{-1}), normalization of ALT (48% vs. 16%), and HBeAg seroconversion (12% vs. 6%). It is also useful for the treatment of lamivudine-resistant HBV infection.

Resistance

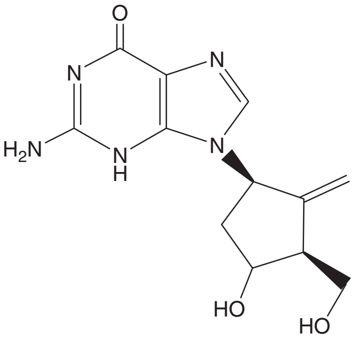
Adefovir resistance occurs in approximately 6% of patients 3 years after adefovir monotherapy. Mutations in the HBV polymerase B domain (A181V/T) and the D domain (N236T) confer resistance to adefovir.

Adverse effects

Nephrotoxicity is the major side effect of higher doses of adefovir. It causes a proximal convoluted tubule lesion characterized by a rise in urea and creatinine. Other dose-related clinical adverse events have been gastrointestinal events, including nausea, anorexia and diarrhea. These are usually mild, intermittent and self-limited without the need for concomitant medications or dose interruption.

Entecavir

Entecavir



Chemistry, mechanism of action, and antiviral activity

Entecavir (2-amino-1,9-dihydro-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one), monhydrate is a guanosine nucleoside analogue.

Entecavir is efficiently phosphorylated by cellular kinases to the active triphosphate metabolite. It affects three-steps in the replication of HBV: (1) prevent the priming of the HBV reverse transcriptase, (2) prevent reverse transcribing of the HBV pregenomic mRNA, and (3) inhibits DNA-dependent DNA synthesis (i.e., terminating viral DNA synthesis). The HBV polymerase binds preferentially to entecavir triphosphate, and entecavir triphosphate does not affect human mitochondrial DNA synthesis. The effect of entecavir on human cellular polymerase is minimal. Studies prior to approval of entecavir for HBV treatment suggested that entecavir did not have anti-HIV activity at clinical relevant concentrations. However, recent studies have suggested an anti-HIV activity of entecavir at drug concentrations in the low nanomolar range.

Entecavir is well absorbed after oral administration achieving peak plasma concentrations between 0.6–1.5 h. Entecavir is not a substrate of the cytochrome P450 (CYP) enzyme system. It is eliminated primarily in the urine through glomerular filtration and tubular secretion. The mean elimination $T_{1/2}$ of entecavir varies from 77 to 149 h in patients with normal function. The intracellular half-life of the triphosphate metabolite *in vitro* studies is about 15 h.

Clinical indications

Entecavir was approved in March 2005, for the management of adult patients with chronic HBV infection who have active viral replication and/or elevation in liver transaminases or signs of active disease on histological examination. In phase III trials, responses achieved with entecavir surpassed previously published response rates for IFN-α-2b, lamivudine, and adefovir dipivoxil. With recent reports of an anti-HIV activity of entecavir, entecavir monotherapy probably should not be used in individuals with HIV–HBV coinfection who need HBV but not HIV treatment.

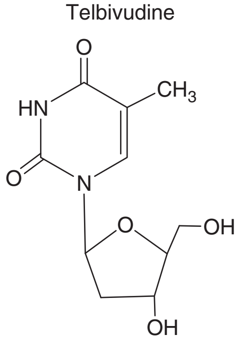
Resistance

The prevalence rate of resistance to entecavir in HBV-treatment naive is about 1.2%. However, virologic rebound and resistance have been reported in 43% of lamivudine-resistant patients after 4 year of switching treatment to entecavir. Entecavir resistance requires the following amino acid sequence changes in the reverse transcriptase domain of HBV; M204V/I + L184G, S202I, or M250V.

Adverse effects

Most adverse events in the phase III studies were mild and comprised of headache, upper respiratory tract infections, cough, fatigue, pharyngitis, abdominal pain, and gastrointestinal upset. The most common laboratory abnormality was ALT level greater than five times the upper limit of normal. Monitoring for long-term toxicity is needed.

Telbivudine



Chemistry, mechanism of action, and antiviral activity

Telbivudine (β-L-2′-deoxythymidine) is an L-configured nucleoside with potent and specific activities against HBV and other hepadnaviruses. Telbivudine is a competitive inhibitor of both HBV viral reverse transcriptase and DNA polymerase. Telbivudine is phosphorylated by cellular kinases to the triphosphate metabolite, which competes with naturally occurring thymidine triphosphate for viral DNA elongation. The incorporation of telbivudine into the viral DNA terminates viral DNA chain elongation. In contrast to other nucleoside analogue, such as lamivudine, telbivudine preferentially inhibits anticomplement or second-strand DNA, whereas lamivudine triphosphate preferentially inhibits the complement DNA synthesis.

Preliminary studies have shown a potent inhibition of HBV replication with a safe profile and no effect on mitochondrial metabolism. Telbivudine triphosphate does not inhibit human cellular polymerase α, β, or γ. In addition, telbivudine triphosphate is not a substrate for human DNA polymerase and thus will not induce genotoxicity.

Telbivudine is rapidly absorbed after oral dosing with peak plasma concentration achieved within 1–3 h, the absolute oral bioavailability of telbivudine is not known. Over an 8-h period, telbivudine exhibits an apparent single-phase decline, with $T_{1/2}$ of 2.5–5 h. However, a presence of a second, slower elimination phase was observed with intensive sampling in healthy volunteers up to 168 h post-dosing. The second phase starts approximately 16–24 h after dosing, with a long observed terminal-phase $T_{1/2}$ of approximately 40 h. The long plasma $T_{1/2}$ of telbivudine is consistent with the long intracellular $T_{1/2}$ (14 h) of its triphosphate *in vitro* studies. The elimination $T_{1/2}$ of telbivudine increases with renal dysfunction, therefore, dosage reduction of telbivudine is recommended in individuals with renal dysfunction.

Clinical indications

Telbivudine was approved in October 2006 by the FDA for treatment of chronic HBV infection. In clinical trails with primary end point of therapeutic response (a composite of suppression of HBV DNA and either loss of serum HBeAg or ALT normalization) after one year, in HBeAg-positive patients a therapeutic response occurred in 75% of patients treated with telbivudine and 67% of those treated with lamivudine. In HBeAg-negative patients, the response was 75 and 77% for telbivudine and lamivudine, respectively. In the second year of the study, telbivudine was found to be superior to lamivudine. Using the two drugs in combination was no more effective than telbivudine monotherapy.

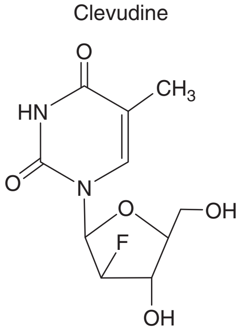
Resistance

HBeAg-positive, 21.6%, and HBeAg-negative, 8.6%, recipients of telbivudine had HBV DNA rebound that was associated with resistance mutations. Lamivudine-resistance HBV strains have a high level of cross resistance to telbivudine. The mutations in the RT domain of HBV associated with telbivudine resistance are M204I or M204I + L180I/V.

Adverse effects

Most of the adverse effects of telbivudine reported in clinical studies were mild to moderate. The most common were elevated creatinine phosphokinase (CPK), an enzyme present in muscle tissue and a marker for the breakdown of muscle tissue, upper respiratory tract infection, fatigue, headache, abdominal pain, and cough.

Clevudine



Clevudine was recently approved in South Korea for the treatment of hepatitis B after demonstration of potent anti-hepatitis B activity in phase II and III clinical trials. It is likely to be licensed for hepatitis B treatment in other countries.

Chemistry, mechanism of action, and antiviral activity

Clevudine [1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl) thymidine] is a nucleoside analogue of the unnatural β-L configuration with potent activity against HBV and some activity against EBV. Clevudine is efficiently phosphorylated by cellular kinases to clevudine-triphosphate in target cells. The mechanism of action is mainly inhibition of viral plus-strand DNA synthesis. Preclinical studies revealed that human cellular DNA polymerases α, β, γ, and δ could not utilize the 5′-triphosphate of clevudine as a substrate and, hence, the lack of cytotoxicity. The EC_{50} of clevudine for HBV inhibition values ranges from 0.02 to 0.84 $\mu mol\,l^{-1}$. Clevudine is well absorbed after oral administration with estimated long half-life of 44–60 h.

Clinical indications

Clevudine is approved for treatment of chronic hepatitis B infection in South Korea. In a randomized, placebo-controlled phase III study in South Korea, chronic HBeAg-positive patients who received 30 mg of clevudine once daily for 24 weeks maintained a 3.73 $\log_{10}$ and 2.02 $\log_{10}$ viral suppression at 34 and 48 weeks, respectively. A unique characteristic of clevudine is the slow rebound of viremia after cessation of treatment.

Resistance

In vitro studies suggest that there may be cross-resistance with lamivudine-resistant HBV mutants. In animal studies resistance occurred in the B domain of the polymerase gene, within 12 months of treatment.

Adverse effects

In clinical trials, clevudine was well tolerated without any serious adverse events reported. Long-term toxicity has to be closely monitored.

Future Prospects

Current antiviral agents either inhibit hepatitis B replication, or invoke an immune response, which may be necessary but not sufficient to effect viral control. Moreover, antiviral resistance remains a concern with long-term therapy, the search for novel agents, and treatment strategies with minimal or no resistance and good long-term safety profile are the focus of ongoing research. Tenofovir, and emtricitabine, licensed for the treatment of HIV infections, also have activity against HBV, but are not yet FDA-approved for this indication. There are a number of new nucleoside and nucleotide analogue in the pipeline; elvucitabine, valtorcitabine, amdoxovir, racivir, MIV 210, β-L-FddC, alamifovir and hepavir B may soon be part of the armamentarium for hepatitis B treatment.

Another challenge is the management of hepatitis B in individuals with HIV coinfection. Appropriate combination regimens for individuals with coinfections are expected in the near future; target treatment of HBV to alter the outcome and take into account the impact of HBV treatment on HIV.

Therapeutics for Papillomavirus

HPVs are small DNA viruses with strict epithelial tropism. HPV infection induces the hyperproliferation of epithelial cells, leading to a broad spectrum of human diseases, ranging from benign warts (self-limiting) to malignant neoplasms. In general, there is no virus-specific effective systemic therapy available. Furthermore, treatment of disease with current therapies has not been shown to reduce the rates of transmission.

The recently FDA-approved quadrivalent prophylactic vaccine (HPV6/11/16/18) has been shown in clinical trials to be effective in preventing high-grade vulval and vaginal lesion associated with HPV 16 and 18. With time, this prophylactic vaccine is expected to reduce the incidence of HPV infections, particularly, infections due to the vaccine types (HPV6, 11, 16, and 18).

Interferon

IFNs have antiproliferative, antiviral, and immunomodulatory properties. IFNs have been administered (mostly IFN-α) topically, systemically, and intralesionally with variable results. They are more effective if used in combination with either local surgery or podophyllotoxin. Several large controlled trials have demonstrated inconsistent clinical benefits of the use of standard IFN-α therapy of condyloma acuminatum (caused by HPV) that was refractory to cytodestructive therapies. Intralesional therapy is painful, systemic therapy is associated with influenza-like symptoms such as fever and myalgia. Furthermore, IFN treatment is expensive and there is limited efficacy.

Imiquimod

This is an immunomodulator approved by the FDA for topical treatment of external and perianal genital warts. It acts as a ligand for Toll-like receptor 7 and activates macrophage and dendritic cells to release IFN α and other proinflammatory cytokines. With imiquimod application, gradual clearance of warts occurs in about 50% of patients over an average of 8–10 weeks. The adverse effects are; application site reactions (irritation, pruritus, flaking, and erosion), and systemic effects including fatigue and influenza-like illness.

Podophyllotoxin

Podophyllotoxin is the main cytotoxic ingredient of podophyllin, a resin used for many years for topical treatment of warts. The exact mechanism of action is unknown. Podophyllotoxin 0.5% solution or gel is similar in effectiveness to imiquimod but may have more adverse effects. Adverse effects include irritation of the adjacent skin, local erosion, ulceration and scarring.

Trichloroacetic acid, podophyllotoxin, and cryotherapy (with liquid nitrogen or a cryprobe) remain the most widely used treatments for external genital warts, but response rate is only 60–70%, and at least 20–30% of responders will have recurrence.

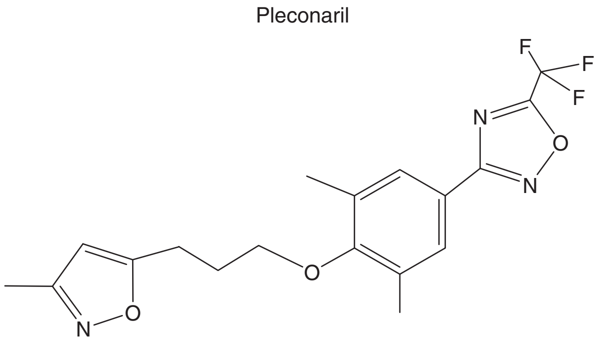
Future Prospects

The current therapies are not targeted antiviral therapies. They result in the physical removal of the lesion or the induction of nonspecific inflammation, thereby inducing a bystander immune response. There is urgent need to develop specific and effective antiviral agents for HPV infections.

Therapeutics for Enteroviral Infections

The enteroviruses include nearly 70 serotypes of closely related pathogens that cause a wide spectrum of human illness, from mild nonspecific fever to common upper respiratory infections, aseptic meningitis, severe myocarditis, encephalitis, and paralytic poliomyelitis. Certain patients, including antibody-deficient individuals, bone marrow recipients, and neonates, may develop potentially life-threatening enterovirus infections for which therapeutic options have been limited. There are case series of the use of immune serum globulin and pleconaril for serious enteroviral infections. Pleconaril failed to secure FDA approval because of its induction of CYP 3A enzyme activity, and the potential for drug interactions, particularly the interference with oral contraceptives.

Pleconaril



Chemistry, mechanism of action, and antiviral activity

Pleconaril (3-{3,5-dimethyl-4-[[3-methyl-5-isoxazolyl}-propyl]phenyl]-5[trifluoromethyl]-1,2,4-oxadiazole) exerts its antiviral effect by integrating into a hydrophobic pocket inside the virion, and prevents viral replication by inhibiting viral uncoating and blocking viral attachment to host cell receptors, thus interrupting the infection cycle. The viral capsid structure, which is the target of pleconaril, is relatively conserved among the picornaviruses. Pleconaril has broad spectrum and potent activity against enteroviruses and rhinoviruses.

Pleconaril is 70% bioavailability when given orally. This high level of bioavailability was achieved by the substitution of trifluoromethyl on the oxadiazole ring that reduces its degradation in the liver by enzymes involved in oxidative processes. The metabolic stabilization is reflected in the drug's long serum half-life (about 6.5 h) after oral dosing. Pleconaril also readily penetrates the blood-brain barrier.

Clinical indications

Common cold

In a phase I trial of pleconaril for treatment of common cold, there was a significant reduction in rhinorrhea of about 1.5 days in those on 400 mg three times daily, and a reduction in a severity score as compared to the placebo. Subsequent trials confirmed a modest reduction in length of symptoms for common cold in patients treated with pleconaril.

Immunocompromised host

Patients with compromised humoral immunity, such as those with agammaglobulinemia, who contract enteroviral infections may develop chronic meningitis and meningoencephalitis, often with a fatal outcome. There are case reports of the efficacy of pleconaril in these patients.

Enteroviral meningitis

For treatment of enteroviral meningitis, two large studies showed a marginal statistical improvement in a clinical score in the pleconaril-treated groups. A subsequent small study of 21 infants with proven enteroviral meningitis in the United States did not have enough power to show unequivocal benefit with pleconaril.

Resistance

Resistance to pleconaril has been reported in some serotypes of enteroviruses, however, the mechanism is not well understood.

Adverse effects

Pleconaril is generally well tolerated. The most common adverse events are headache, diarrhea, and nausea. Long-term use of pleconaril is associated with an increase in menstrual irregularities in women.

Future Prospects

Pleconaril has not been licensed for treatment of enteroviral infections; there is an urgent need to identify alternative drugs that might be effective. There are several investigational compounds; however, none has reached phase I clinical trial. Combinations of drugs are likely to offer the best chance of cure and protection from enterovirus infections in the future.

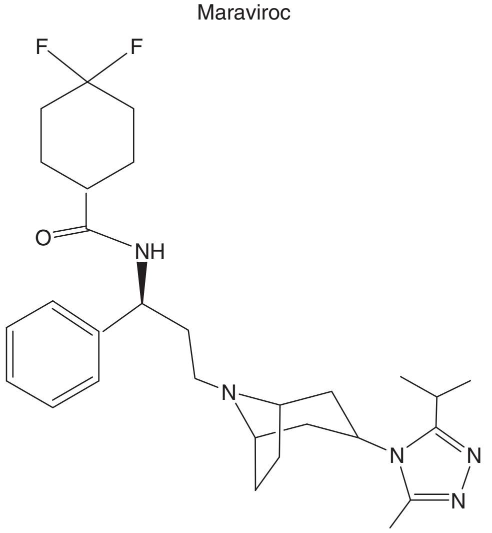
Anti-HIV Agents

The combination of three or more anti-HIV agents into multidrug regimens, often termed highly active antiretroviral therapy (HAART), can efficiently inhibit HIV viral replication to achieve low or undetectable circulatory HIV-1 levels. This is the start-of-the-art treatment of AIDS or HIV-infected individuals. Drug combinations are, in principle, aimed at obtaining synergism between the compounds, while reducing the likelihood of the development of drug resistance virus, and minimizing toxicity. The available anti-HIV drugs are categorized according the step they target within the HIV viral life cycle (**Figure 2**); (1) binding inhibitors, for example, coreceptor antagonist (maraviroc); (2) fusion inhibitors (enfuvirtide); (3) reverse transcriptase inhibitors (nucleoside/nucleotide (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine, and tenofovir), and non-nucleoside (nevirapine, delavirdine, and efavirenz) analogue); (4) integrase inhibitors (raltegravir); and (5) protease inhibitors (saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, fosamprenavir, lopinavir, atazanavir, tipranavir, and darunavir).

Fixed-dose combinations and once-daily dosage forms of many anti-HIV agents are available. There are fixed-dose combinations for zidovudine/lamivudine, zidovudine/lamivudine/abacavir, abacavir/lamivudine, tenofovir/emtricitabine, and tenofovir/emtricitabine/efavirenz.

Coreceptor Antagonist

Maraviroc



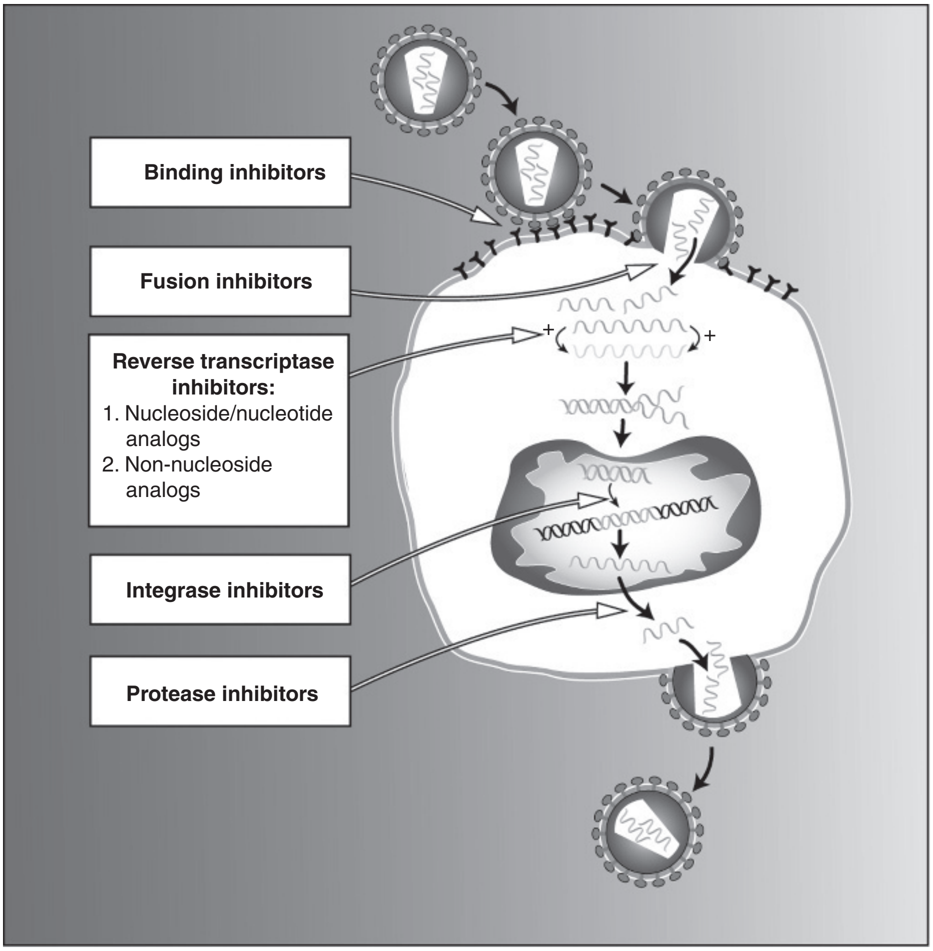

Binding inhibitors
Fusion inhibitors
Reverse transcriptase inhibitors:
1. Nucleoside/nucleotide analogs
2. Non-nucleoside analogs
Integrase inhibitors
Protease inhibitors
+
+

Figure 2 HIV life cycle showing the stages of intervention of available anti-HIV agents.

Chemistry, mechanism of action, and antiviral activity

Maraviroc (4,4-difluoro-*N*-{(1*S*)-3-[*exo*-3-(3-isopropyl-5-methyl-4*H*-1,2,4 triazol-4-yl)-8-azabicyclo(3,2,1]oct-8-yl]-1-phenylpropyl}cyclohexanecarboxamide) is the first of the class of CCR5 coreceptor antagonists licensed (August 2007) for HIV treatment. Maraviroc selectively binds to the human chemokine receptor CCR5 present on the cell membrane, preventing the interaction of HIV-1 gp120 and CCR5 necessary for CCR5-tropic HIV-1 to enter cells. It inhibits the replication of CCR5-tropic laboratory strains and primary isolates of HIV-1 *in vitro*. The mean EC_{50} for maraviroc against various strains of HIV-1 ranges from 0.1 to 1.25 $nmol\,l^{-1}$ (0.05 to 0.64 $ng\,ml^{-1}$) in cell culture. Maraviroc was not active against CXCR4-tropic and dual-tropic viruses (EC50 value >10 $\mu mol\,l^{-1}$). The antiviral activity of maraviroc against HIV-2 has not been evaluated.

The absolute bioavailability for 100 and 300 mg doses are 23 and 33%, respectively. Peak plasma concentrations of maraviroc are attained at 0.5–4 h following single oral dose of 1200 mg administered to uninfected volunteers. Maraviroc is bound (approximately 76%) to human plasma proteins. It is principally metabolized by the cytochrome P450 system to metabolites that are essentially inactive against HIV-1. Maraviroc is a substrate of CYP3A and the efflux transporter P-glycoprotein (Pgp), and therefore, its pharmacokinetics are likely to be modulated by inhibitors and inducers of these enzymes/transporters. The terminal half-life in healthy subjects is 14–18 h.

Clinical indications

Maraviroc is approved for use in combination with other anti-HIV agents for the treatment of adults with CCR5-tropic HIV-1, who are treatment-experienced with evidence of viral replication and HIV-1 strains resistant to multiple antiretroviral agents.

Resistance

The resistance profile in treatment-naive and treatment-experienced subjects has not been fully characterized. HIV-1 variants with reduced susceptibility to maraviroc have been selected in cell culture, following serial passage of two CCR5-tropic viruses (CC1/85 and RU570). The maraviroc resistant viruses remained CCR5-tropic with no evidence

Table 1 The three-letter and one-letter codes for amino acid residues

Amino acid	*Three-letter code*	*One-letter code*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

of a change from a CCR5-tropic virus to a CXCR4-using virus. Two amino acid residue substitutions (**Table 1**, letter codes of amino acids) in the V3-loop region of the HIV-1 envelope glycoprotein (gp160), A316T and I323V were shown to be necessary for the maraviroc-resistant phenotype in the HIV-1 isolate CC1/85. In the RU570 isolate, a 3-amino acid residue deletion in the V3 loop, ΔQAI (HXB2 positions 315–317), was associated with maraviroc resistance. The clinical relevance of these mutations is not known.

Adverse effects

The most common adverse events reported with maraviroc were cough, fever, upper respiratory tract infections, rash, musculoskeletal symptoms, abdominal pain, and dizziness. The product label includes a warning about liver toxicity (hepatoxicity) and a statement about the possibility of heart attacks.

Fusion Inhibitors

Enfuvirtide

Chemistry, mechanism of action, and antiviral activity

Enfuvirtide, a linear 36-amino acid synthetic peptide with the N-terminus acetylated and the C-terminus is a carboxamide, is the first licensed agent in the class of fusion inhibitors. Enfuvirtide interferes with the entry of HIV-1 into cells by inhibiting fusion of viral and cellular membranes (**Figure 2**). Enfuvirtide binds to the first heptad-repeat (HR1) in the gp41 subunit of the viral envelope glycoprotein and prevents the conformational changes required for the fusion of viral and cellular membranes. The IC_{50} of enfuvirtide for baseline clinical isolates ranged from 0.089 to 107 $\mathrm{nmol\,l^{-1}}$ (0.4 to 480 $\mathrm{ng\,ml^{-1}}$). Enfuvirtide is active against R5, X4, and dual tropic viruses, but has no activity against HIV-2.

Enfuvirtide is administered twice daily by subcutaneous injection. Single-dose vials contain 108 mg of enfuvirtide for the delivery of approximately 90 $\mathrm{mg\,ml^{-1}}$ when reconstituted. The absolute bioavailability is 84.3 ± 15.5%. Following 90 mg bid dosing of enfuvirtide subcutaneously in combination with other antiretroviral agents in HIV-1 infected subjects, the median $T_{\max}$ was 4 h (ranged from 4 to 8 h). Enfuvirtide is catabolized by proteolytic enzymes. It is not metabolized by hepatic CYP450 isoenzyme systems. There are no known clinically significant interactions between enfuvirtide and other medications.

Clinical indications

Enfuvirtide was approved by the FDA in March 2003 for use in adults, and in children aged 6 and older, with advanced HIV infection. Enfuvirtide is used with other anti-HIV agents to treat HIV-1 infection in patients who are treatment-experienced and have detectable viral loads even though they are taking anti-HIV agents.

Resistance

HIV-1 isolates with reduced susceptibility to enfuvirtide have been selected *in vitro*. Genotypic analysis of these resistant isolates showed mutations that resulted in amino acid substitutions at the enfuvirtide binding HR1 domain positions 36–38 of the HIV-1 envelope glycoprotein gp41. In clinical trials, HIV-1 isolates with reduced susceptibility to enfuvirtide have been recovered from subjects failing enfuvirtide-containing regimen. Most of the isolates with decreased in susceptibility to enfuvirtide of greater than fourfold exhibited genotypic changes in the codons encoding gp41 HR1 domain amino acids 36–45.

HIV-1 clinical isolates resistant to nucleoside analogue reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, and protease inhibitors are susceptible to enfuvirtide in cell culture.

Adverse effects

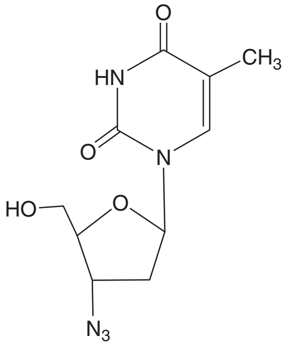
The most common adverse effects of enfuvirtide are injection site reactions. Other symptomatic side effects may include insomnia, headache, dizziness, and nausea. Several cases of hypersensitivity have been described. In phase III studies, bacterial pneumonia was seen at a higher rate in patients who received enfuvirtide than in those who did not receive enfuvirtide. Eosinophilia is the primary laboratory abnormality seen with enfuvirtide administration.

Reverse Transcriptase Inhibitors

Nucleoside/nucleotide reverse transcriptase inhibitors

Zidovudine

Zidovudine



Chemistry, mechanism of action, and antiviral activity Zidovudine (3′-azido-2′,3′-dideoxythymidine) is a pyrimidine analogue with an azido group substituting for the 3′ hydroxyl group on the ribose ring. Zidovudine is initially phosphorylated by cellular TK and then to its diphosphate by cellular thymidylate kinase. The triphosphate derivative competitively inhibits HIV reverse transcriptase, and functions as a chain terminator. Zidovudine inhibits HIV-1 at concentrations of approximately 0.013 μg ml^{-1}. In addition, it inhibits a variety of other retroviruses. Synergy has been demonstrated against HIV-1 when zidovudine is combined with didanosine, zalcitabine, lamivudine, nevirapine, delavirdine, saquinavir, indinavir, ritonavir, and other compounds. It was the first drug to be licensed for the treatment of HIV infection, and still is used in combination with other drugs as initial therapy for some patients.

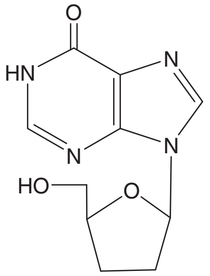
Zidovudine is available in capsule, syrup, and intravenous formulations. Oral bioavailability is approximately 65%. Peak plasma levels are achieved approximately 0.5–1.5 h after treatment. Zidovudine penetrates cerebrospinal fluid, saliva, semen, and breast milk and it crosses the placenta. Drug is predominately metabolized by the liver through the enzyme uridine diphosphoglucuronosyltransferase to its major inactive metabolite 3′-azido-3′-deoxy-5′-*O*-β-D-glucopyranuronosylthymidine. The elimination $T_{1/2}$ is approximately 1 h; however, it is extended in individuals who have altered hepatic function.

Clinical indications Zidovudine is used in combination with other anti-HIV agents. It is administered orally at 600 mg day^{-1} (300 mg tablet, twice a day). The single most important usage of zidovudine in the last decade has been the peripartum three-part zidovudine regimen, which has decreased the incidence of transmission of HIV infection from pregnant women to their infants.

Adverse effects The predominant adverse effect of zidovudine is myelosuppression, as evidenced by neutropenia and anemia, occurring in 16 and 24% of the patients, respectively. Zidovudine has been associated with skeletal and cardiac muscle toxicity, including polymyositis. Nausea, headache, malaise, insomnia, and fatigue are common side effects.

Didanosine

Didanosine



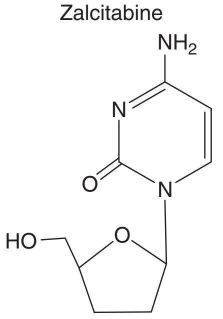
Chemistry, mechanism of action, and antiviral activity Didanosine (2′,3′-dideoxyinosine) is a purine nucleoside with inhibitory activity against both HIV-1 and HIV-2. Didanosine is activated by intracellular phosphorylation. It is first converted to 2′,3′-dideoxyinsine-5′-monophosphate by 5′nucleotidase and inosine 5′-monophosphate phosphotransferase and subsequently to 2′, 3′-dideoxyadenosine-5′-monophosphate by adenylsuccinate synthetase and lyase. It is then converted to diphosphate by adenylate kinase and subsequently by creatine kinase or phosphoribosyl pyrophosphate synthetase to the triphosphate. The triphosphate metabolite is a competitive inhibitor of HIV reverse transcriptase and a chain terminator. The spectrum of activity of didanosine is enhanced by synergism with zidovudine and stavudine as well as the protease inhibitors.

Didanosine is acid labile and has poor solubility. A buffered tablet results in 20–25% bioavailability. A 300 mg oral dose achieves peak plasma concentrations of 0.5–2.6 μg ml^{-1} with a $T_{1/2}$ of approximately 1.5 h. It is metabolized to hypoxanthine and is cleared primarily by the kidneys.

Clinical indications Didanosine is used in combination with other anti-HIV agents as part of HAART. It is given as two 100 mg tablets (buffered tablets) twice a day or as one 400 mg capsule (delayed-release capsule) once a day.

Adverse effects The most significant adverse effect associated with didanosine therapy is the development of peripheral neuropathy (30%) and pancreatitis (10%). Lipoatrophy, lactic acidosis and diabetes have been observed in patients on antiretroviral regimens containing didanosine.

Zalcitabine



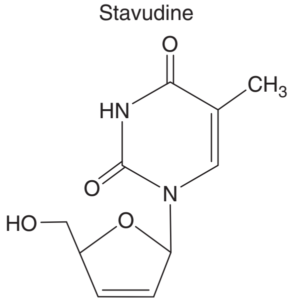
Chemistry, mechanism of action, and antiviral activity Zalcitabine (2′,3′-dideoxycytidine) is a pyrimidine analogue, which is activated by cellular enzymes to its triphosphate derivative. The enzymes responsible for activation of zalcitabine are cell cycle independent, and therefore this offers a theoretical advantage for nondividing cells, specifically dendritic and monocyte/macrophage cells. Zalcitabine inhibits both HIV-1 and HIV-2 at concentrations of approximately 0.03 μmol l^{-1}.

The oral bioavailability following zalcitabine administration is more than 80%. The peak plasma concentrations following an oral dose of 0.03 mg kg^{-1} range from 0.1 to 0.2 μmol l^{-1} and the $T_{1/2}$ is approximately 20 min. The drug is cleared mainly by the kidneys, and therefore, the presence of renal insufficiency leads to a prolong plasma $T_{1/2}$.

Clinical indications Zalcitabine is used as part of HAART regimen for HIV-1 infections. It is administered orally at 2.25 mg day^{-1} (one 0.75 mg tablet every 8 h).

Adverse effects Peripheral neuropathy is the major toxicity associated with zalcitabine administration, occurring in approximately 35% of individuals. Pancreatitis can occur, but does so infrequently. Thrombocytopenia and neutropenia are uncommon (5% and 10%, respectively). Other zalcitabine-related side effects include nausea, vomiting, headache, hepatotoxicity, and cardiomyopathy.

Stavudine



Chemistry, mechanism of action, and antiviral activity Stavudine (2′,3′-didehydro, 3′-deoxythymidine) is a thymidine analogue with significant activity against HIV-1, having inhibitory concentrations, which range from 0.01 to 4.1 μmol l^{-1}. Its mechanism of action is similar to that of zidovudine.

The oral bioavailability of stavudine is more than 85%. Peak plasma concentrations of approximately 1.2 μg ml^{-1} are reached within 1 h of dosing at 0.67 mg kg^{-1} per dose. Stavudine penetrates CSF and breast milk. It is excreted by the kidneys unchanged and, in part, by renal tubular secretion.

Clinical indications Stavudine is used for HIV infection in combination with other anti-HIV agents. Stavudine is a highly potent inhibitor of HIV-1 replication *in vitro*. However, its use has been limited by delayed toxicity, notably peripheral neuropathy and myopathy caused by mitochondrial damage. It is administered orally at 80 mg day^{-1} (one 40 mg capsule every 12 h).

Adverse effects The principal adverse effect of stavudine therapy is the development of peripheral neuropathy. The development of this complication is related to both dose and duration of therapy. Inhibition of mitochondrial DNA synthesis is proposed to induce depletion of cellular mitochondrial DNA and it is ultimately responsible for the delayed toxicity observed with the use of stavudine and other nucleoside reverse transcriptase inhibitors (NRTIs). Neuropathy tends to appear after 3 months of therapy and resolves slowly with medication discontinuation. Other side effects are uncommon. Fatal and nonfatal pancreatitis have occurred during therapy when stavudine was part of a combination regimen that included didanosine. Redistribution and accumulation of body fat (lipoatrophy) have been observed in patients receiving stavudine as part of their antiretroviral regimen.

Lamivudine

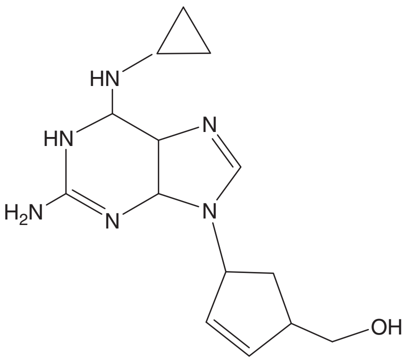
Chemistry, mechanism of action, and antiviral activity The chemistry and mechanism of action of lamivudine have been described previously in section 'Lamivudine' under 'Therapeutics for Hepatitis'. Lamivudine has significant activity *in vitro* against both HIV-1 and HIV-2, as well as HBV. Lamivudine is a competitive inhibitor of the viral reverse transcriptase.

Clinical indications Lamivudine is used in combination with other anti-HIV agents. Lamivudine is given orally at 300 mg day^{-1} (one 150 mg tablet twice a day, or one 300 mg tablet once a day). It is also formulated in combination with zidovudine, or with zidovudine and abacavir as fixed-dose combination tablet.

Adverse effects Lamivudine has an extremely favorable toxicity profile. This may largely be attributed to the low affinity of lamivudine for human DNA polymerases, and the lack of active lamivudine metabolites in the mitochondrial compartment of cells. At the highest doses of 20 mg kg^{-1} day^{-1}, neutropenia is encountered but at a low frequency. In pediatric studies, pancreatitis and peripheral neuropathies have been reported.

Abacavir

Abacavir



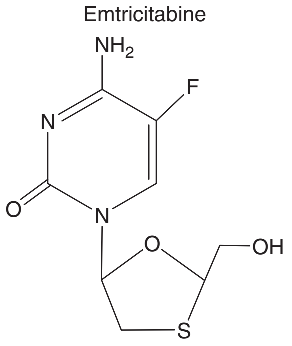
Chemistry, mechanism of action, and antiviral activity Abacavir sulfate, (1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol, is a structural analogue of the purine guanine. The phosphorylation pathway of abacavir differs from that of all other nucleoside analogues. The first step in the conversion of abacavir to its active metabolite, carbovir triphosphate, is phosphorylation to abacavir monophosphate by adenosine phosphotransferase. This step is followed by deamination by a cytosolic enzyme to form carbovir monophosphate, which undergoes two subsequent phosphorylations, to the diphosphate by guanylate kinase and to the triphosphate by nucleoside diphosphate kinase and other enzymes. Carbovir triphosphate competes with endogenous 2′-dGTP for incorporation into the nucleic acid chain, and after incorporation, terminates DNA chain elongation. Abacavir exhibits potent *in vitro* antiviral activity against wild-type HIV-1 (IC_{50} 4.0 μmol l^{-1}), but this activity is lower than the activity of zidovudine (IC_{50} 0.040 μmol l^{-1}). However, there is no significant difference between the levels of activity of abacavir (IC_{50} 0.26 μmol l^{-1}) and AZT (IC_{50} 0.23 μmol l^{-1}) against clinical isolates of HIV-1.

Abacavir is well absorbed after oral administration with a bioavailability between 76 and 96%. After single or multiple doses, $C_{\max}$ is attained after a mean of 0.7–1.7 h, and the mean half-life is 0.8–1.5 h. However, at a dose of 300 mg twice daily as part of a combination regimen, levels of intracellular carbovir triphosphate ranged from <20 to 374 fmol per 10^6 cells. The intracellular carbovir triphosphate was measurable throughout the 24-h study period, with the highest levels found between 6 and 8 h. This finding suggests a long half-life for carbovir triphosphate within cells. The main route of excretion is renal.

Clinical indications Abacavir is used in combination with other anti-HIV agents. It is given as a 300 mg tablet twice a day, or two 300 mg tablets once a day. A fixed-dose combination with lamivudine is available (600 mg abacavir, plus 300 mg lamivudine).

Adverse effects The abacavir hypersensitivity reaction is a potentially fatal syndrome occurring in approximately 5% of HIV-infected patients exposed to this nucleoside analogue after a median of 11 days (range: 1–318 days). Systemic manifestations can include fever, rash, fatigue, and gastrointestinal or respiratory symptoms. Rechallenge with abacavir in individuals presumed to have abacavir hypersensitivity reaction is avoided due to reports of fatal reactions caused by repeated administration of abacavir following a hypersensitivity reaction. The presence of HLA-B∗5701 has been associated with elevated odds of developing abacavir hypersensitivity reaction.

Emtricitabine



Chemistry, mechanism of action, and antiviral activity Emtricitabine, 5-fluoro-1-(2*R*,5*S*)-[2-(hydroxymethyl)-1,3-oxathiolan 5-yl]cytosine, is a fluorinated nucleoside analogue of cytosine. Emtricitabine, similar in many ways to lamivudine, has *in vitro* activity against HIV-1 that is similar to or approximately fourfold to tenfold more potent than that of lamivudine. The EC_{50} for emtricitabine is in the range of 0.0013–0.64 $\mu mol\,l^{-1}$ (0.0003–0.158 $\mu g\,ml^{-1}$) for laboratory and clinical isolates of HIV-1 in cell culture. Against HIV-2, the EC_{50} ranges from 0.007 to 1.5 $\mu mol\,l^{-1}$. The cellular enzymes involved in the phosphorylation of emtricitabine are similar to that of lamivudine. The active triphosphate competitively inhibits reverse transcriptase by being incorporated into the viral genome, and causing termination in DNA chain elongation.

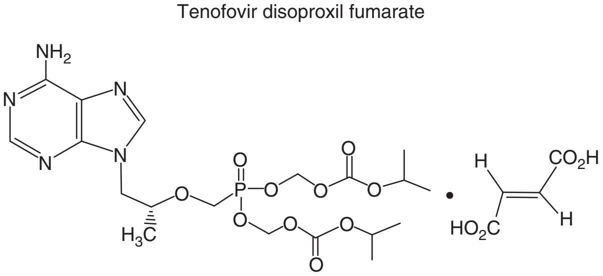
The bioavailability of the capsules and oral solution are 93 and 75%, respectively. Emtricitabine is rapidly and extensively absorbed following oral administration with peak plasma concentrations occurring at 1–2 h post dose.

The mean plasma elimination half-life of emtricitabine after a single dose is about 8–10 h in HIV-infected patients. However, after multiple doses of the drug at a dose of 200 mg daily, the intracellular half-life is approximately 39 h. The high intracellular levels of emtricitabine triphosphate achieved are associated with better suppression of plasma HIV RNA. Emtricitabine is not an inhibitor of human CYP450 enzymes. Emtricitabine is eliminated by a combination of glomerular filtration and active tubular secretion.

Clinical indications Emtricitabine is used with other anti-HIV agents. It is administered orally at a once-daily 200 mg capsule. Emtricitabine is a component of Truvada (a fixed-dose combination of emtricitabine and tenofovir disoproxil fumarate), and Atripla (a fixed-dose combination of efavirenz, emtricitabine, and tenofovir disoproxil fumarate).

Adverse effects The most common adverse events are headache, diarrhea, nausea, and rash, which are generally of mild to moderate severity. Approximately 1% of patients discontinued participation in clinical trials due to these events.

Tenofovir disoproxil fumarate



Chemistry, mechanism of action, and antiviral activity Tenofovir disoproxil fumarate is (a prodrug of tenofovir), 9-[(*R*)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl]methoxy]propyl]adenine fumarate (1:1), converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analogue of adenosine 5′-monophosphate. Tenofovir disoproxil fumarate requires initial diester hydrolysis for conversion to tenofovir and subsequent phosphorylations by cellular enzymes to form tenofovir triphosphate. Tenofovir triphosphate inhibits the activity of HIV-1 reverse transcriptase by competing with the natural substrate deoxyadenosine 5′-triphosphate and, after incorporation into DNA, by DNA chain termination. Tenofovir triphosphate is a weak inhibitor of mammalian DNA polymerases α, β, and mitochondrial DNA polymerase γ. Tenofovir has antiviral activity *in vitro* against HIV-1 with IC_{50} values ranging from 0.5 to 2.2 $\mu mol\,l^{-1}$. The IC_{50} values of tenofovir against HIV-2 range from 1.6 to 4.9 $\mu mol\,l^{-1}$.

The oral bioavailability of tenofovir in fasting patients is approximately 25%. Following oral administration of a single dose of 300 mg to HIV-1 infected patients, maximum serum concentrations are achieved in 1.0 ± 0.4 h. C_{max} and AUC values are 296 ± 90 $ng\,ml^{-1}$ and 2287 ± 685 $ng\,h\,ml^{-1}$, respectively. The oral bioavailability increases when tenofovir is administered after a high-fat meal (40–50% fat). *In vitro* studies indicate that neither tenofovir disoproxil nor tenofovir are substrates of CYP450 enzymes. Tenofovir is primarily excreted by the kidneys by a combination of glomerular filtration and active tubular secretion. The pharmacokinetics of tenofovir is altered in patients with renal impairment.

Clinical indications Tenofovir is used in combination with other anti-HIV agents for the treatment of HIV-1 infection. It is administered orally once daily, 300 mg tablet.

Adverse effects The most common adverse reactions seen in a double-blind comparative controlled study were mild to moderate gastrointestinal events and dizziness. However, the following adverse events have been identified during post-approval use of tenofovir; allergic reaction, hypophosphatemia, lactic acidosis, dyspnea, abdominal pain, increased amylase, pancreatitis, increased liver enzymes, hepatitis, renal insufficiency, renal failure, fanconi syndrome, proximal tubulopathy, proteinuria, increased creatinine, acute tubular necrosis, and nephrogenic diabetes insipidus.

Resistance to nucleoside/nucleotide analogue

Nucleoside analogue-associated mutations (NAMs) develop by at least three pathways: (1) accumulation of zidovudine or thymidine analogue resistance mutations (TAMs), (e.g., 41L, 67N, 70R, 210W, 215Y/F, and 219Q/E); (2) selection of the key 151M mutation, followed by the mutations 62V, 75I, 77L, and 116Y, referred to as the 151 complex; and (3) the 69 insertion complex, consisting of a mutation at codon 69 (typically Ser), followed by an insertion of two or more amino acids (e.g., Ser-Ser, Ser-Arg, or Ser-Gly) and generally accompanied by other NAMs. In clinical isolates, two TAM pathways have been observed: 41L, 210W, 215Y/F and 67N, 70R, 219Q/E/N/R; of these, the 41–210–215 combination is the most prevalent. The cytidine analogue select for the M184V mutation (lamivudine, and emtricitabine), while the K65R is seen with tenofovir selection pressure.

Resistance patterns with earlier nucleoside analogue combinations (zidovudine or stavudine with lamivudine) The resistance profiles seen with earlier nucleoside analogue combinations are well characterized. With thymidine-based NRTIs (lamivudine/zidovudine or stavudine/lamivudine), the M184V mutation emerges rapidly, whereas TAMs are slower to arise. The use of emtricitabine in place of lamivudine would presumably yield similar results, although clinical data are limited. The M184V mutation has been shown to increase zidovudine susceptibility in the absence or presence of zidovudine resistance mutations and without regard to which TAM combination is present. The M184V mutation may increase sensitivity to tenofovir.

The most commonly observed mutations with zidovudine/didanosine or stavudine/didanosine are TAMs. TAMs and multinucleoside resistance (MNR) mutations (Q151M, T69 insert) are more prevalent in didanosine-containing regimens than in lamivudine-containing regimens.

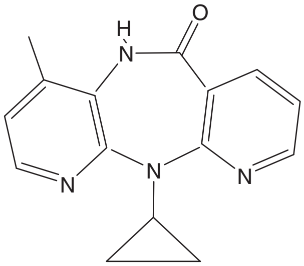
Resistance patterns with new nucleoside/nucleotide analogue combinations With abacavir/lamivudine as backbone, the most common mutation selected for is M184V/I followed by the L74V mutation at treatment failure. The K65R is the major mutation selected *in vitro* by tenofovir alone or in combination with abacavir or lamivudine while abacavir appears to favor L74V and K65R.

In triple-nucleoside/nucleotide regimens (tenofovir/lamivudine/didanosine or tenofovir/lamivudine/abacavir) that lack a thymidine analogue, early treatment failure has been associated with a high frequency of M184V. In addition, 50% of the patients with M184V also harbor the K65R.

Non-nucleoside reverse transcriptase inhibitors

Nevirapine

Nevirapine



Chemistry, mechanism of action, and antiviral activity Nevirapine (11-cyclopropyl-5,11-dihydro-4-methyl-6*H*-dipyrido[3,2-b:2′,3′-e]; [1,4]diazepin-6-one) is a reverse transcriptase inhibitor of HIV-1. However, its mechanism of action is different from nucleoside analogue. It binds to a hydrophobic pocket adjacent to the active site of the reverse transcriptase and causes conformational changes that affect replication. Nevirapine has a bioavailability of approximately 65%. Peak serum concentration of 3.4 $\mu g\,ml^{-1}$ is achieved approximately 4 h after a 400 mg oral dose. Nevirapine is metabolized by liver microsomes to hydroxymethyl-nevirapine.

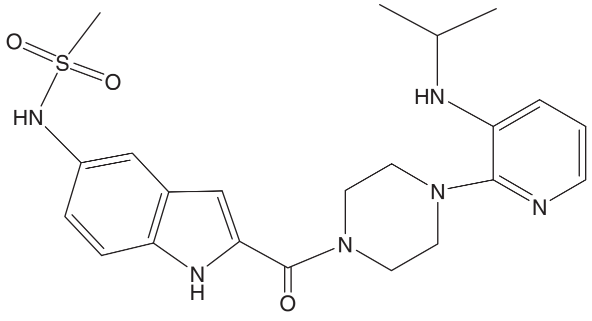
Clinical indications Nevirapine is used in combination with other anti-HIV agents. It is administered orally at 200 mg day^{-1} for the first 14 days (one 200 mg tablet per day), then 400 mg day^{-1} (two daily 200 mg tablets). Single-dose nevirapine is used widely in resource-limited settings to prevent mother-to-child transmission of HIV infection.

Adverse effects The most common adverse effects include the development of a nonpruritic rash in as many as 50% of patients who received 400 mg day^{-1}. In addition, fever, myalgias, headache, nausea, vomiting, fatigue, and diarrhea have also been associated with administration of drug.

Resistance Changes in two sets of amino acid residues (100–110 and 180–190) in the reverse transcriptase gene confer resistance to nevirapine. Nevirapine monotherapy is associated with resistance most frequently appearing at codon 181.

Delavirdine

Delavirdine



Chemistry, mechanism of action, and antiviral activity Delavirdine (1-[5-methanesulfonamido-1*H*-indol-2-yl-carbonyl]-4-[3-(1-methylethylamino) pyridinyl] piperazine) is a second-generation bis (heteroaryl) piperazine licensed for the treatment of HIV infection. Its mechanism of action is similar to that of nevirapine. It is absorbed rapidly when given orally with bioavailabilty of >60%. Delavirdine is metabolized by the liver with an elimination $T_{1/2}$ of approximately 1.4 h. It has an inhibitory concentration against HIV-1 of approximately 0.25 µmol l^{-1}. Inhibitory concentrations for human DNA polymerases are significantly higher.

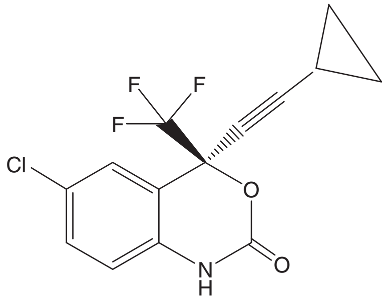
Clinical indications Delavirdine is used in combination with other anti-HIV agents. It is administered at 1200 mg day^{-1} (two 200 mg tablets three times a day).

Adverse effects Delavirdine administration is associated with a maculopapular rash. Other side effects are less common.

Resistance Delavirdine resistance can be generated rapidly both *in vitro* and *in vivo* with the codon change identified at 236, resulting in an increase and susceptibility to >60 µmol l^{-1}. Delavirdine resistance can be conferred by mutations at codons 181 and 188, as seen with other non-nucleoside analogue.

Efavirenz

Efavirenz



Chemistry, mechanism of action, and antiviral activity Efavirenz [(*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4 (trifluoromethyl)-2*H*-3,1-benzoxazin-2-one] is a non-NRTI that can be administered once daily. Activity is mediated predominately by noncompetitive inhibition of HIV-1 reverse transcriptase. HIV-2 reverse transcriptase, and human cellular DNA polymerases α, β, γ, and δ are not inhibited by efavirenz. The 90–95% inhibitory concentration of efavirenz is approximately 1.7–25 nmol l^{-1}.

Clinical indications Efavirenz is used in combination with other antiretroviral agents for the treatment of HIV-1 infection. Combination therapy has resulted in a 150-fold or greater decrease in HIV-1 RNA levels.

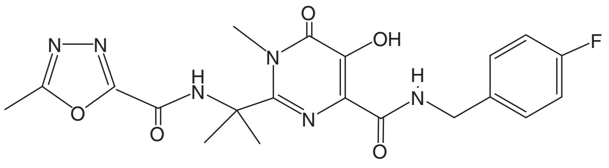
Adverse effects The most common adverse events are skin rash (25%), which is associated with blistering, moist desquamation, or ulceration (1%). In addition, delusions and inappropriate behavior have been reported in 1 or 2 patients per 1000.

Resistance Resistance to efavirenz is caused by mutation in the reverse transcriptase gene as with other non-nucleoside analogue, and appears rapidly.

Integrase Inhibitors

Raltegravir

Raltegravir



Chemistry, mechanism of action, and antiviral activity

Raltegravir, a structural analogue of a class of compounds with a distinct diketo acid moiety, is a novel HIV-1 integrase inhibitor with potent *in vitro* activity against HIV-1 (IC$_{95}$ of 33 nmol l^{-1}) in the presence of 50% human serum. It is active against a wide range of wild-type and multidrug-resistant HIV-1 clinical isolates and has potent activity against viruses that use CCR5 and/or CXCR4 coreceptors for entry.

Raltegravir is absorbed rapidly, with median $T_{\max}$ values in the fasting state of about 1 h; plasma concentrations decrease from $C_{\max}$ in a biphasic manner, with a half-life of approximately 1 h for the initial (α) phase and an apparent half-life of approximately 7–12 h for the terminal (β) phase. The pharmacokinetic data for raltegravir are supportive of twice daily administration. It is metabolized by hepatic glucuronidation and has no effect on CYP3A4. Approximately 7–14% of the raltegravir dose is excreted unchanged in urine.

Clinical indications

Raltegravir received priority approval from the FDA (October 2007) for treatment of HIV-1 infection in combination with other antiretroviral agents in treatment-experienced patients with evidence of HIV-1 replication despite ongoing antiretroviral therapy. The dosage of raltegravir is 400 mg administered orally, twice daily with or without food.

Adverse effects

Side effects (mostly mild to moderate) were seen with similar frequency in the raltegravir and placebo arms; the rate of serious adverse events was less than 3% across arms. No lipid abnormalities have been reported so far with raltegravir.

Resistance

In 9 of 41 patients failing raltegravir, no mutations were detected; while in 32 of 41 patients, three mutational patterns were described (N155H, Q148K/R/H, and, rarely, Y143R/C). The clinical implications of these findings are unknown.

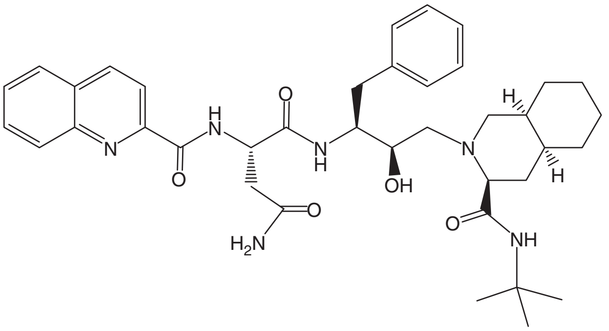
Protease Inhibitors

Protease inhibitors are used in combination with other anti-HIV agents for treatment of HIV infection. They are a potent component of HAART regimens. Protease inhibitors are used in combination with ritonavir as the boosting protease inhibitor. The concept of boosting involves pharmacokinetic drug interactions; currently available protease inhibitors are metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. Ritonavir is the most powerful enzyme inhibitor in the protease inhibitor class. The combination with ritonavir allows the boosted protease inhibitor to maintain prolonged blood levels. This allows for decreased dosage, and reduces a three times a day schedule to a twice daily or even a once daily regimen.

Long-term HAART containing protease inhibitors has been most strongly associated with syndromes characterized by dyslipidemia, peripheral lipodystrophy, and insulin resistance.

Saquinavir

Saquinavir



Chemistry, mechanism of action, and antiviral activity

Saquinavir (*cis*-*N*-tert-butyl-decahydro-2[2(*R*)-hydroxy-4-phenyl-3-(*S*)-([*N*-(2 quinolycarbonyl)-Lasparginyl] amino butyl)-4a*S*, 8a*S*]-isoquinoline-3[S]-carboxyamide methanesulfonate) is a hydroxyethylamine-derived peptidomimetic HIV protease inhibitor. Saquinavir inhibits HIV-1 and HIV-2 at concentrations of 10 nmol l^{-1} and is synergistic with other nucleoside analogue as well as selected protease inhibitors.

Oral bioavailability is approximately 30% with extensive hepatic metabolism. Peak plasma concentrations of 35 mg μl^{-1} are obtained following a 600 mg dose.

The clinical efficacy of saquinavir is limited by poor oral bioavailability but improved formulation (soft-gel capsule) enhances efficacy. Saquinavir is boosted with 100 mg twice a day of ritonavir to improve its bioavailabilty and efficacy even against saquinavir-resistant HIV strains.

Adverse effects

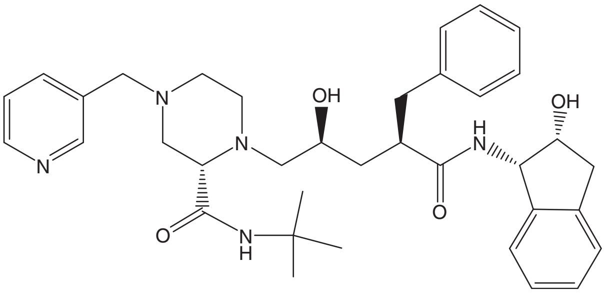
Adverse effects are minimal with no dose-limiting toxicities. Abdominal discomfort, including diarrhea and nausea, has been reported infrequently.

Resistance

Mutations at codon sites 90 and 48 of the protease gene result in approximately a 30-fold decrease in susceptibility to saquinavir.

Indinavir

Indinavir



Chemistry, mechanism of action, and antiviral activity

Indinavir {*N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-(1,1-dimethylethlaminocarbonyl)-4-(pyridin-3-yl) methylpiperazin-1-yl]-4[*S*]-hydroxy-2[*R*]-phenylmethyl pentanamide} is a peptidomimetic HIV-1 and HIV-2 protease inhibitor. At concentrations of 100 nmol l^{-1}, indinavir inhibits 90% of HIV isolates. Indinavir is rapidly absorbed with a bioavailability of 60% and achieves peak plasma concentrations of 12 µmol l^{-1} after a 800 mg oral dose.

Adverse effects

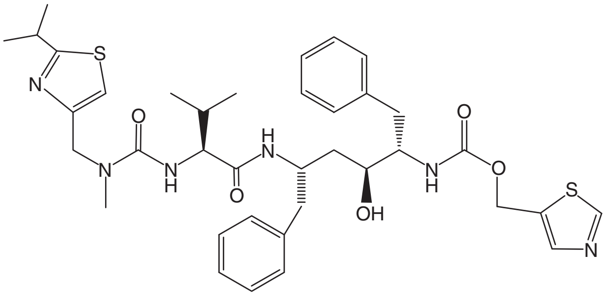
Although indinavir is well tolerated, commonly encountered adverse effects include indirect hyperbilirubinemia (10%) and nephrolithiasis (5%).

Resistance

Indinavir resistance develops rapidly with monotherapy and occurs at multiple sites. The extent of resistance is directly related to the number of codon changes in the HIV protease gene. Codon 82 is a common mutation in indinavir-resistant HIV isolates.

Ritonavir

Ritonavir



Chemistry, mechanism of action, and antiviral activity

Ritonavir (10-hydroxy-2-methyl-5[1-methylethyl]-1[2-(1-methylethyl)-4-thiazo lyl]-3,6,dioxo-8,11-bis[phenylmethyl]-2,4,7,12-tetra azatridecan-13-oic-acid, 5 thiazolylmethylester, [5S-(5R, 8R,10R, 11R)]) is an HIV protease inhibitor with activity *in vitro* against HIV-1 laboratory strains (0.02–0.15 µmol l^{-1}). It is synergistic when administered with nucleoside analogue. Oral bioavailability is approximately 80%, with peak plasma levels of approximately 1.8 µmol l^{-1} after 400 mg administered every 12 h. The plasma half-life is approximately 3 h.

Adverse effects

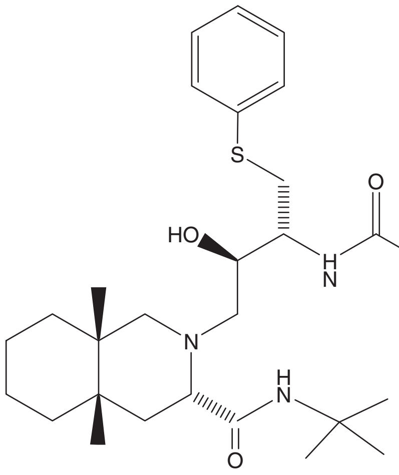
Adverse effects include nausea, diarrhea, and headache, but all occur at a low frequency.

Resistance

Ritonavir has cross-resistance to indinavir. Mutations at codon 82 are the most common.

Nelfinavir

Nelfinavir



Chemistry, mechanism of action, and antiviral activity

Nelfinavir [3*S*-(3*R*, 4a*R*, 8a*R*, 22′*S*, 3′*S*)]-2-[2″-hydroxy-3′-phenylthiomethyl-4′-aza-5′-ox-o-5′-(2″methyl-3′-hydroxyphenyl)pentyl]-decahydroiso-quinoline-3-*N*-(tert-butyl-carboxamide methanesulfonic acid salt) is another peptidomimetic HIV protease inhibitor. Inhibitory concentrations of HIV-1 are in the range of 20–50 nmol l^{-1}. It has anti-HIV-2 activity. Nelfinavir is orally bioavailable at approximately 40%, achieving peak plasma concentrations of 2 or 3 mg following a 800 mg dose every 24 h. The drug is metabolized by hepatic microsomes.

Adverse effects

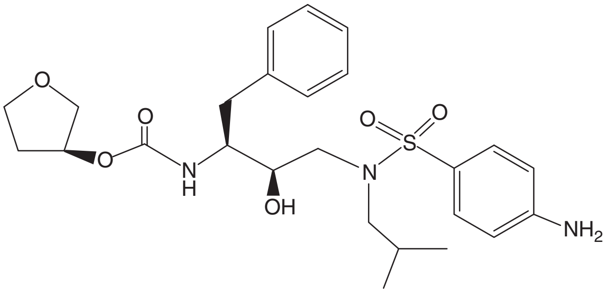
Nelfinavir is well tolerated with mild gastrointestinal complication reported.

Resistance

Cross-resistance to other protease inhibitors, particularly saquinavir, indinavir, or ritonavir, is not common. The most frequently demonstrated site of mutation is at codon 30.

Amprenavir

Amprenavir



Chemistry, mechanism of action, and antiviral activity

Amprenavir is a hydroxyethylamine sulfonamide peptidomimetric with a structure identified as (3*S*)-tetrahydro-3-furyl *N*-(1*S*,2*R*)-3-(4-amino-*N* isobutylbenzenesulfonamido)-1-benzyl-2-hydroxypropylcarbamate. It is active at a concentration of 10–20 nmol l^{-1}. The oral bioavailability is >70% and peak plasma concentrations of 6.2–10 µg ml^{-1} are achieved after dosages of 600–1200 mg. The plasma half-life is 7–10 h. CSF concentrations are significant. Amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system.

Adverse effects

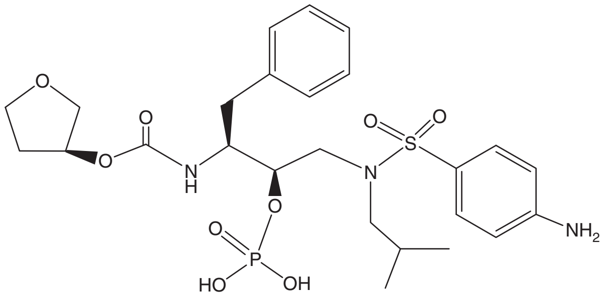
The most common adverse events are gastrointestinal events (nausea, vomiting, diarrhea, and abdominal pain/discomfort), which are mild to moderate in severity. Also, skin rash can occur in patients on amprenavir.

Resistance

Genotypic analysis of isolates from treatment-naive patients failing amprenavir-containing regimens showed mutations in the HIV-1 protease gene resulting in amino acid substitutions primarily at positions V32I, M46I/L, I47V, I50V, I54L/M, and I84V, as well as mutations in the p7/p1 and p1/p6 Gag and Gag-Pol polyprotein cleavage sites.

Fosamprenavir

Fosamprenavir



Chemistry, mechanism of action, and antiviral activity

Fosamprenavir, a prodrug of amprenavir [(3*S*)-tetrahydrofuran-3-yl (1*S*,2*R*)-3-[[(4-aminophenyl) sulfonyl](isobutyl) amino]-1 benzyl-2-(phosphonooxy) propylcarbamate monocalcium salt], is an inhibitor of human HIV protease. Fosamprenavir is rapidly hydrolyzed to amprenavir by enzymes in the gut epithelium. After administration of a single dose of fosamprenavir to HIV-1-infected patients, the peak concentration occurs between 1.5 and 4 h (median 2.5 h). Amprenavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The plasma elimination half-life of amprenavir is approximately 7.7 h.

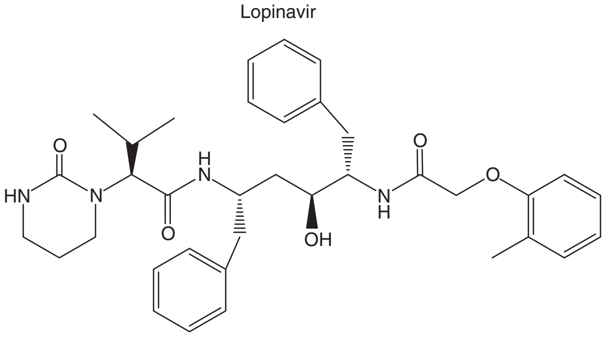
Adverse effects

Side effects profile is similar to that of amprenavir.

Resistance

Fosamprenavir selects for amprenavir-associated mutations on treatment failure, though, at a much lower incidence.

Lopinavir



Chemistry, mechanism of action, and antiviral activity

Lopinavir [N-(4(S)-(2-(2,6-dimethylphenoxy)-acetylamino)-3(S)-hydroxy-5-phenyl-1(S)-benzylpentyl)-3-methyl-2(S)-(2-oxo(1,3-diazaperhydroinyl)butanamin)] is an inhibitor of the HIV protease, prevents cleavage of the Gag-Pol polyprotein, resulting in the production of immature, noninfectious viral particles. It is coformulated with ritonavir at 4:1 ratio (Kaletra). In the presence of 50% human serum, the mean EC_{50} values of lopinavir against HIV-1 laboratory strains ranges from 65 to 289 nmol l^{-1} (0.04–0.18 μg ml^{-1}). It has some activity against HIV-2 strains. Lopinavir peak plasma concentration occurs approximately 4 h after administration. Lopinavir is metabolized by CYP3A, and ritonavir inhibits the metabolism of lopinavir, thereby increasing the plasma levels of lopinavir.

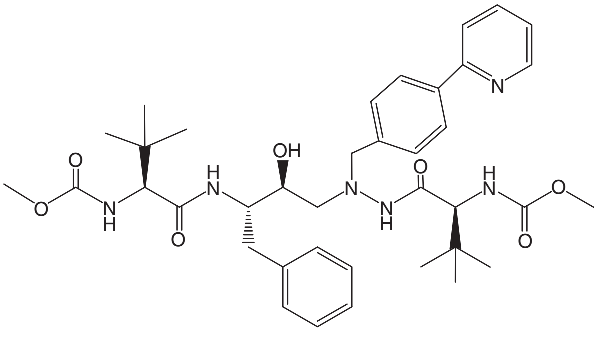
Adverse effects

Most common adverse events are nausea, diarrhea, increased cholesterol and triglycerides, and lipodystrophy.

Resistance

Virologic response to lopinavir/ritonavir has been shown to be affected by the presence of three or more of the following amino acid substitutions in protease at baseline: L10F/I/R/V, K20M/N/R, L24I, L33F, M36I, I47V,G48V, I54L/T/V, V82A/C/F/S/T, and I84V.

Atazanavir



Chemistry, mechanism of action, and antiviral activity

Atazanavir [(3*S*,8*S*,9*S*,12*S*)-3,12-Bis(1,1-dimethylethyl)-8-hydroxy-4,11-dioxo-9 (phenylmethyl)-6-[[4-(2-pyridinyl)phenyl]methyl]-2,5,6,10,13-pentaazatetradecanedioic acid dimethyl ester, sulfate (1:1)] is an azapeptide inhibitor of HIV-1 protease. Atazanavir exhibits anti-HIV-1 activity with an EC_{50} in the absence of human serum of 2–5 nmol l^{-1} against a variety of laboratory and clinical HIV-1 isolates *in vitro.*

Atazanavir is rapidly absorbed with a T_{max} of approximately 2.5 h. Atazanavir is metabolized in the liver by the cytochrome P450 3A4 (CYP3A4) enzyme system. The mean elimination half-life of atazanavir in healthy volunteers and HIV-infected adult patients is approximately 7 h.

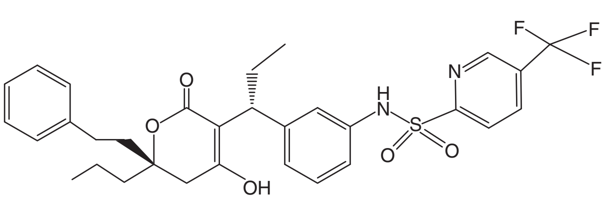
Adverse effects

The most common adverse event in patients is the asymptomatic elevations in indirect (unconjugated) bilirubin related to inhibition of UDP-glucuronosyl transferase (UGT). The hyperbilirubinemia is reversible upon discontinuation of atazanavir. Atazanavir may cause abnormal electrocardiogram findings, increased serum glucose, and lipodystrophy in some patients.

Resistance

HIV-1 isolates with a decreased susceptibility to atazanavir have been selected *in vitro* and obtained from patients treated with atazanavir or atazanavir/ritonavir. The mutations associated with resistance to atazanavir are I50L, N88S, I84V, A71V, and M46I. Atazanavir-resistant clinical isolates from treatment-naive harbored the I50L mutation (after an average of 50 weeks of atazanavir therapy), often, in combination with an A71V mutation. However, the viral isolates with the I50L mutation are phenotypically resistant to atazanavir but show *in vitro* susceptibility to other protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir).

Tipranavir



Chemistry, mechanism of action, and antiviral activity

Tipranavir [2-Pyridinesulfonamide, N-[3-[(1R)-1-[(6R)-5,6-dihydro-4-hydroxy-2-oxo-6-(2-phenylethyl)-6-propyl-2H-pyran-3-yl]propyl]phenyl]-5-(trifluoromethyl)] is a nonpeptidic HIV protease inhibitor belonging to the class of 4-hydroxy-5,6-dihydro-2-pyrone sulfonamides.

Tipranavir inhibits the replication of laboratory strains of HIV-1 and clinical isolates *in vitro*, with EC_{50} ranging from 0.03 to 0.07 $\mu mol\, l^{-1}$ (18–42 $ng\, ml^{-1}$).

The effective mean elimination half-life of tipranavir/ritonavir in healthy volunteers and HIV-infected adult patients is approximately 4.8 and 6.0 h, respectively, at steady state following a dose of 500/200 mg twice daily with a light meal. Tipranavir is predominantly metabolized by the CYP 3A4 enzyme system.

Tipranavir, coadministered with 200 mg of ritonavir, is used in combination with other anti-HIV agents for the treatment of HIV-1 infected adult who are highly treatment-experienced with evidence of viral replication, or have HIV-1 strains resistant to multiple protease inhibitors. Response rates are reduced if five or more protease inhibitor-associated mutations are present at baseline and patients are not given concomitant enfuvirtide with tipranavir/ritonavir.

Adverse effects

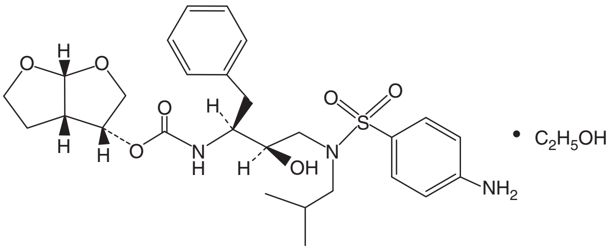
Adverse events include rash, increased cholesterol, increased triglycerides, lipodystrophy, and hepatitis. There have been reports of both fatal and nonfatal intracranial hemorrhage with the use of tipranavir/ritonavir. Tipranavir/ritonavir should be used with caution in patients who may be at risk of increased bleeding from trauma, surgery or other medical conditions, or who are receiving medications known to increase the risk of bleeding such as antiplatelet agents or anticoagulants.

Resistance

HIV-1 isolates that were 87-fold resistant to tipranavir were selected *in vitro* by 9 months and contained 10 protease mutations that developed in the following order: L33F, I84V, K45I, I13V, V32I, V82L, M36I, A71V, L10F, and I54V/T. In clinical trials tipranavir had less than fourfold decreased susceptibility against 90% of HIV-1 isolates resistant to amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, or saquinavir. Tipranavir-resistant viruses selected for *in vitro* have decreased susceptibility to the protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, and ritonavir but remain sensitive to saquinavir.

Darunavir

Darunavir



Chemistry, mechanism of action, and antiviral activity

Darunavir, in the form of darunavir ethanolate, has the following chemical name: [(1*S*,2*R*)-3-[[(4-aminophenyl)-sulfonyl](2-methylpropyl)amino]-2-hydroxy-1-(phenylmethyl) propyl]-carbamic acid (3*R*,3a*S*,6a*R*)-hexahydrofuro[2,3-*b*]furan-3-yl ester monoethanolate. It is an inhibitor of the HIV protease. Darunavir exhibits activity against laboratory strains and clinical isolates of HIV-1 and laboratory strains of HIV-2 with median EC_{50} values ranging from 1.2 to 8.5 $nmol\, l^{-1}$ (0.7–5.0 $ng\, ml^{-1}$). Darunavir, coadministered with 100 mg ritonavir twice daily, was absorbed following oral administration with a T_{max} of approximately 2.5–4 h. The absolute oral bioavailability of a single 600 mg dose of darunavir alone and after coadministration with 100 mg ritonavir twice daily was 37 and 82%, respectively. Darunavir is primarily metabolized by CYP3A. Ritonavir inhibits CYP3A, thereby increasing the plasma concentrations of darunavir when given in combination.

Darunavir, coadministered with 100 mg ritonavir, and with other anti-HIV agents, is indicated for the treatment of HIV infection in antiretroviral treatment-experienced adult patients, such as those with HIV-1 strains resistant to more than one protease inhibitor.

Adverse effects

The most common treatment-emergent adverse events (>10%) reported in the *de novo* subjects, regardless of causality or frequency, were diarrhea, nausea, headache, and nasopharyngitis. Other side effects are increased triglycerides, increased cholesterol, lipodystrophy, increased glucose, and increased liver enzyme levels.

Resistance

Darunavir-resistant virus derived in cell culture from wild-type HIV had 6- to 21-fold decreased susceptibility to darunavir and harbored three to six of the following amino acid substitutions S37N/D, R41E/S/T, K55Q, K70E, A71T, T74S, V77I, or I85V in the protease. In phase IIb trial, the amino acid substitution V32I developed on darunavir/ritonavir (600/100 mg twice a day) in greater than 30% of virologic failure isolates and substitutions at amino acid position I54 developed in greater than 20% of virologic failure isolates. Other substitutions that developed in 10–20% of darunavir/ritonavir virologic failure isolates occurred at amino acid positions I15, L33, I47, G73, and L89.

Future Prospects in HIV Therapeutics

The simplification of HAART regimens has been a high priority for many years. As the number of effective drugs increases, so does the number of possible effective regimens. The trend toward fixed-dose combinations and once-daily dosage forms of many antiretroviral drugs has provided welcome relief to patients. Not only is their medication burden simplified, but as a consequence of improved adherence to therapy, they should experience better control of HIV and thus reduced morbidity.

New drug discovery strategies attempt at circumventing the current drug resistant problem by focusing on either novel targets or new compounds capable of suppressing HIV strains that are resistant to current inhibitors. There are several nucleoside analogues in preclinical and clinical studies. Notably are the novel 4′-substituted thymidine analogues with potent antiviral activity and less cytotoxic. An example is the recently discovered 2′,3′-didehydro-3′-deoxy-4′-ethynylthymidine, structurally related to stavudine, is a more potent inhibitor of HIV-1 replication and is much less inhibitory to mitochondrial DNA synthesis and cell growth in cell cultures than its progenitor stavudine. The triphosphate metabolite accumulates in cells much longer than stavudine, and exerts persistent antiviral activity even after removal of drug from culture. It also has a unique resistance profile when compared to other thymidine analogues and maintains activity against multidrug resistant HIV strains. It is currently in preclinical studies with phase I and II clinical trials anticipated in 2008. Other new NRTIs in phase II clinical trials are MIV-310, SPD754, and L-d4FC. A new non-nucleoside analogue in phase II trials is TMC125, it appears to have potent antiviral activity in treatment-experienced patients with resistance mutations to this drug class or in patients who are treatment naive. There is increasing number of compounds discovered as anti-HIV agents targeted at virtually any step in the replicative cycle of the virus and novel targets in development.

Summary

It is anticipated that new and effective treatments for viral infections will be available with the advent of modern and improved technology, based on molecular biology, combinatorial chemistry, and computer-aided design of compounds with greater specificity targeting on viral life cycle.

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Archaea (overview)

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Defining Statement
Introduction
Historical Recognition
Archaeal Ecology and Environmental Biology
Novel Molecular and Genetic Characteristics of Archaea
Archaeal Genomics
Biotechnological Applications of Archaea
Conclusion
Further Reading

Glossary

Archaea One of three phylogenetic groups, or domains, of life (along with the Bacteria and Eucarya) at the highest level, above kingdom (or phylum). Archaeal cells are morphologically prokaryotic cells and lack nuclei.

Bacteria One of three phylogenetic groups, or domains, at the highest level. Bacterial cells are prokaryotic and lack nuclei, like the Archaea, and in contrast to Eucarya.

chaperones Molecular machines that assist and control the process of protein folding and turnover in the cell.

crenarchaeota A phylogenetic group, or kingdom, of the Archaea characterized by many hyperthermophilic isolates.

DNA replication Process of copying parental strands of DNA to daughter strands, using DNA polymerases and other accessory proteins that initiate, prime, elongate, and terminate the process.

Eucarya One of three phylogenetic groups, or domains, of life at the highest level that possesses cells with nuclei and includes all eukaryotes (plants, animals, fungi, and protozoa).

euryarchaeota A phylogenetic group, or kingdom, of the Archaea characterized by all halophiles and methanogens, and some thermophiles and hyperthermophiles.

extremophiles Microorganisms that grow and flourish in environments inhospitable for the growth of most other organisms. Includes many but not all Archaea.

genomics An approach to studying organisms by determining the complete sequence of a genome followed by computational and functional analysis of the encoded genes.

haloarchaea Halophilic microorganisms belonging to the archaeal domain.

halophiles Salt-loving organisms, which grow in highly saline environments, usually containing salt concentrations in excess of sea salinity up to saturation.

mesophiles Organisms that grow optimally in the middle temperature range, usually defined by the ambient temperature on Earth's surface (~20–42 °C).

metagenomics Large-scale sequencing of DNA isolated or cloned from microbial communities in the environment, which may be analyzed for genes and genomes using computational analysis.

methanogens Anaerobic microorganisms that use simple organic and inorganic materials, such as acetate, hydrogen, and carbon dioxide for their metabolism, and generate methane as the primary end product.

orthologues Genes or proteins with identical or very similar functions in different organisms inferred from phylogenetic or experimental studies.

phylogeny Inference of evolutionary relationships among microorganisms based on the extent of nucleotide or amino acid sequence differences among orthologues.

prokaryotes Microorganisms that do not possess a membrane-bounded nucleus, including all members of both the Archaea and Bacteria.

psychrophiles Cold-loving organisms that grow optimally at low temperatures, usually with an optimum between 0 and 20 °C.

thermophiles Heat-loving organisms that grow optimally at elevated temperatures, usually with temperature optima above 50 °C; hyperthermophiles grow optimally above 80 °C.

transcription The process of synthesizing messenger RNA from DNA using RNA polymerase and other accessory proteins such as initiation and termination factors.

translation The process of decoding messenger RNA into proteins using the protein synthesis machinery, including ribosomes, aminoacyl–tRNA synthetases, and other factors.

Abbreviations

ANME	anaerobic methane-oxidizing Archaea
GINS	Go, Ichi, Nii, and San
MCM	minichromosome maintenance
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
rRNA	ribosomal RNA
TBP	TATA-binding protein
TFB	transcription factor IIB
tRNA	transfer RNA

Defining Statement

Archaea are prokaryotic microorganisms that are members of the third domain of life, distinct from Bacteria and Eucarya. Archaea dominate many extreme environments and are widespread in many common environments, including the mammalian gastrointestinal tract. Their information transfer systems (DNA replication, transcription, and translation) are simplified versions of their eucaryal counterparts.

Introduction

Archaea are prokaryotic microorganisms that are members of the third branch (or domain) of life, distinct from the other two domains – Bacteria and Eucarya. Archaea were recognized as a coherent group in the tree of life using small ribosomal RNA (rRNA) sequence comparisons by C. R. Woese and coworkers in 1977. Archaea have been detected in nearly all environments examined using culture-independent molecular techniques, including 16S rRNA sequencing. However, most well-characterized Archaea have been cultured from extreme environments that are very salty, acidic, alkaline, hot, cold, or anaerobic where they are sometimes dominant. Methanogenic Archaea have been detected in the mammalian gastrointestinal tract, but no archaeal species causing disease has been identified thus far. Many Archaea are chemoautotrophs and can grow on simple inorganic chemicals, others are heterotrophs and grow on complex organic materials, and a few have phototrophic capabilities and can use light energy for growth. Although Archaea are prokaryotic in their morphology, consisting of cells bounded by a single lipid membrane and lacking a nucleus, some of their molecular characteristics are similar to nucleated eucaryal cells. Archaeal genomes are 0.5–5.75 Mbp circles and include genes for information transfer machineries (DNA replication, transcription, and translation) that are simplified versions of their eucaryal counterparts. They also have unique features, like their membrane lipids, which contain branched chain isoprenoid units in fatty chains linked to a glycerol-1-phosphate head group via ether linkages. Archaea have many useful qualities that have been translated into applications in biotechnology, including thermostable DNA polymerases for polymerase chain reaction (PCR).

Historical Recognition

Many microorganisms studied by early microbiologists were Archaea, but their fundamental distinction from common Bacteria escaped notice until the pioneering phylogenetic work of C. R. Woese and coworkers in the latter third of the twentieth century (**Figure 1**). The metabolic activities of microorganisms that would later be called Archaea were evident well before the invention of the microscope, with anaerobic methanogenic species giving rise to marsh gas (combustible air), described as early as the time of the Roman empire, and salt-loving archaeal halophiles producing red and pink hues in hypersaline ponds used to harvest salt from the sea, described in ancient texts. By contrast, hyperthermophilic archaeal species, some of which grow optimally above 100 °C, near hydrothermal marine vents located in trenches kilometers below the ocean surface, were not discovered until the late twentieth century.

After the advent of microscopes in the fifteenth century, prokaryotic microorganisms became collectively known as ‘bacteria’, a scientific term originally introduced by C. G. Ehrenberg in 1838. A role for microbes in methanogenesis was first described by A. Béchamp in 1868, but their classification as Archaea required an additional century. The isolation of pure species of methanogens from complex microbial communities was finally accomplished in the mid-twentieth century through the work of microbiologists, H. A. Barker, K. Schnellen, and T. C. Stadtman and followed the development of specialized anaerobic microbiological methodology. Some of the earliest named methanogenic species were *Methanobacterium formicicum*, *Methanococcus vannielii*, and *Methanosarcina barkeri*.

A second major group of Archaea, the halophilic Archaea (or haloarchaea), were identified as agents of food

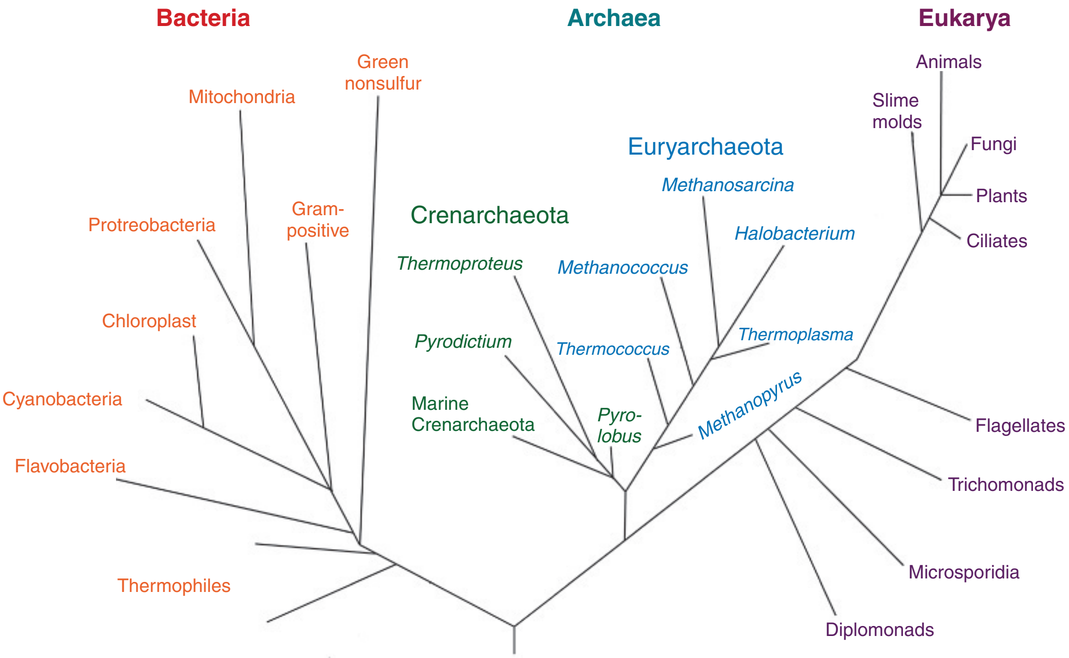
Bacteria
Archaea
Eukarya
Green nonsulfur
Mitochondria
Protreobacteria
Gram-positive
Chloroplast
Cyanobacteria
Flavobacteria
Thermophiles
Crenarchaeota
Thermoproteus
Pyrodictium
Marine Crenarchaeota
Pyro-lobus
Euryarchaeota
Methanosarcina
Halobacterium
Methanococcus
Thermococcus
Thermoplasma
Methanopyrus
Animals
Slime molds
Fungi
Plants
Ciliates
Flagellates
Trichomonads
Microsporidia
Diplomonads

Figure 1 An evolutionary tree, emphasizing the three-domain view of life. Within domain Archaea, two kingdoms (Euryarchaeota and Crenarchaeota) and representative genera are shown.

spoilage, when salting was widely used for preserving fish and meats before the advent of refrigeration. In the early twentieth century, haloarchaeal isolates were named *Bacillus halobius ruber* and *Bacterium halobium* by H. Klebahn and H. F. M. Petter, respectively, and subsequent isolates were named *Halobacterium halobium* (*Halobacterium salinarium*), *Halobacterium cutirubrum*, and *Halobacterium salinarum*. Taxonomy of these halophilic Archaea continues to be controversial, especially since the order, family, and certain genera include the term bacterium rather than archaeum. In 1968, W. Stoeckenius discovered that these *Halobacterium* species contain a light-driven proton pump in a specialized region of the membrane, termed purple membrane. Many have the ability to produce buoyant gas vesicles for flotation, the combination of which gives these microorganisms the ability to grow phototrophically.

Studies of membrane lipids in the 1960s by M. Kates and coworkers showed that those present in many halophiles and methanogens were of unusual composition. They found glycerol-1-phosphate as the head group and ether bonds linking hydrocarbon side chains with methyl-branched isoprenoids (**Figure 2**), as opposed to the glycerol-3-phosphate head group and typical ester bond linkages to straight-chain acyl group fatty acids found in most other organisms. Subsequently, it became clear that these unusual characteristics of membrane lipids were also shared with some thermophilic species, suggesting a common relationship between these metabolically diverse microorganisms. However, the surprising discoveries of lipid chemistry, though widely employed at present, were not originally used for taxonomic or phylogenetic grouping, or to propose novel evolutionary relationships among microorganisms.

With the advent of molecular biology in the 1970s, it became possible to recognize Archaea as a distinct phylogenetic group. Woese and his students intensively studied small rRNAs, molecules that form the catalytic center of the protein synthesis machinery, carrying out a process common to all known free-living organisms. Woese reasoned that since all known organisms contain

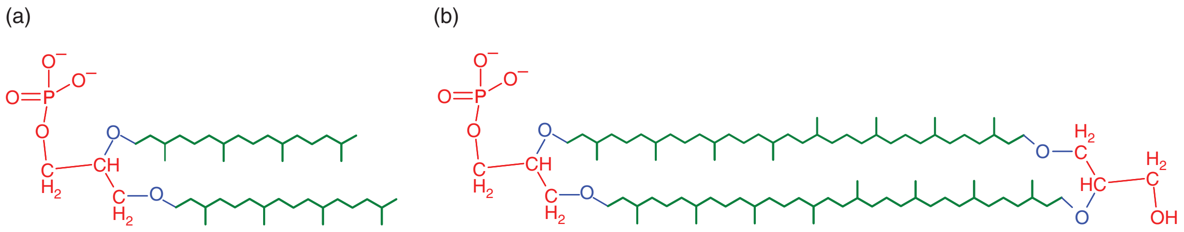
(a)
(b)
O⁻
O=P
O
C
H2
CH
C
H2
O
OH
HC

Figure 2 Characteristic archaeal lipids. (a) Typical diphytanyl glycerol diether phospholipid without a head group. (b) Dibiphytanyl glycerol tetraether lipid common to thermophiles. In both panels, the red portions highlight the glycerol-1-phosphate backbone (stereoisomer used in Bacteria and Eucarya), the blue portions highlight the ether bonds, and the green portions highlight the isoprenoid hydrocarbon chains.

small rRNAs (either 16S or 18S), and as their central functions are evolving slowly, they would serve as the ideal molecular chronometer to infer deep evolutionary relationships. Using RNA fingerprinting (catalogs of RNA oligonucleotides generated with sequence-specific nucleases), a deep division was discovered among prokaryotic species, with the common bacterial species grouping together on the one hand (dubbed '*eu*bacteria' or *true* bacteria) and uncommon species inhabiting diverse environments (named '*archae*bacteria' or *ancient* bacteria) on the other. This classification was confirmed by sequence analysis of rRNAs and their genes, and in 1990 Woese, O. Kandler, and M. Wheelis proposed the new names – domain Archaea, Bacteria, and Eucarya – to emphasize the existence of three fundamentally different types of organisms in the tree of life (**Figure 1**).

The last two decades of the twentieth century brought about exciting developments in archaeal research. Many new thermophilic species were identified by W. Zillig, K. O. Stetter, and others, especially after the discovery by H. Jannasch and M. J. Mottl of microbial communities near deep-sea hydrothermal vents (**Figure 3**). While the original thermophilic Archaea available for molecular phylogenetic analysis included only species of *Thermoplasma* and *Sulfolobus*, growing in the 55–80 °C temperature range, hyperthermophilic species (growing best above 80 °C or in cases of high-pressure environments, even above 100 °C) became known through both culture-dependent and culture-independent techniques. For example, *Methanocaldococcus* (formerly *Methanococcus*) *jannaschii* and a handful of other microorganisms became the first free-living organisms to have their genomes completely sequenced,

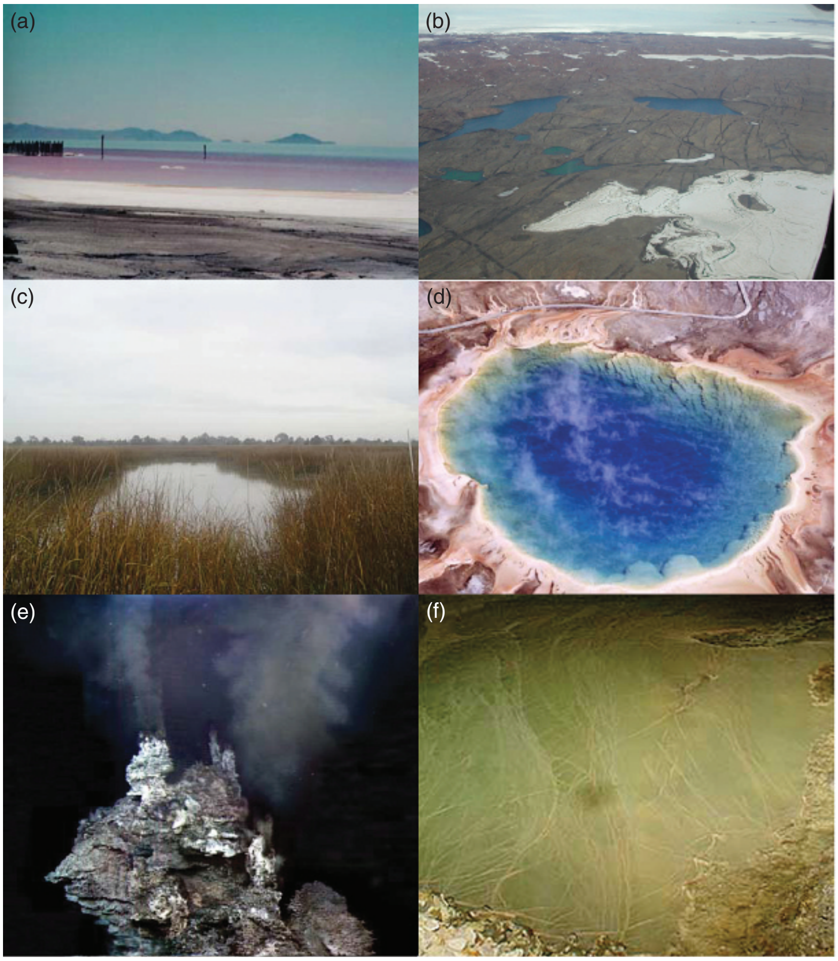

Figure 3 Archaeal habitats. (a) Bloom of haloarchaea, including *Halobacterium* sp., in the north arm of Great Salt Lake, Utah is visible as red brine. Photo courtesy of S DasSarma. (b) Antarctic lakes, for example, Deep Lake, in the Vestfold Hills where the psychrophile, *Halorubrum lacusprofundi*, was isolated. Photo courtesy of Australian Antarctic Data Centre. (c) Wetlands and marshes, where methanogens are found and studied. Photo courtesy of W Whitman. (d) Thermophilic mats radiating from Grand Prismatic Spring, Yellowstone National Park. Photo courtesy of Yellowstone National Park. (e) Hydrothermal vents called 'black smokers' in deep-sea marine trenches, like the Godzilla Vent in the Mid-Atlantic Ridge, are a rich source of hyperthermophiles. Photo courtesy of D Kelley. (f) Archaeal acidophilic biofilms seen at the Richmond Mine at Iron Mountain, California. Photo courtesy of J Banfield.

helping to confirm the three-domain view of life. At about the same time, several *Pyrococcus* species yielded novel thermostable polymerases that improved the PCR method, increasing the value of Archaea for biotechnology.

A combination of genome sequencing together with environmental and metagenomic studies has greatly increased our current knowledge of the archaeal domain. As a result, the evolutionary unity of the Archaea and their distinction from the Bacteria and Eucarya are now firmly established, as is the subdivision of Archaea into the kingdoms Crenarchaeota and Euryarchaeota (**Figure 1**). Many studies using orthologous proteins and genes (e.g., collections of all ribosomal proteins) as well as comprehensive sets of predicted proteins from complete genome sequencing have largely confirmed the results of 16S rRNA-based phylogenetic analysis. Relationships between individual species do vary, but they generally reflect differing evolutionary rates and uncertain gene histories, including lateral or horizontal gene transfers. The great diversity of sequences has recently led to the proposal of many new phyla, including two additional kingdoms of Archaea, Korarchaeota, and Nanoarchaeota. However, some of these branches are poorly resolved (**Figure 4**), indicating that further detailed studies are necessary to shed light on the true diversity of existent archaeal phyla and taxa.

A great deal of interest has recently focused on the nature of the earliest evolutionary events giving rise to the three domains of life. Most phylogenetic studies are consistent with Bacteria branching earliest from the ancestral cell or progenote near the root of the tree of life, with a common ancestor to both Archaea and Eucarya diverging later to form these two domains (**Figure 1**). However, this hypothesis is not universally accepted, and many different scenarios have been proposed to account for these groups. Some investigators have even proposed that the more complex Eucarya, which have nuclei surrounded by membrane and

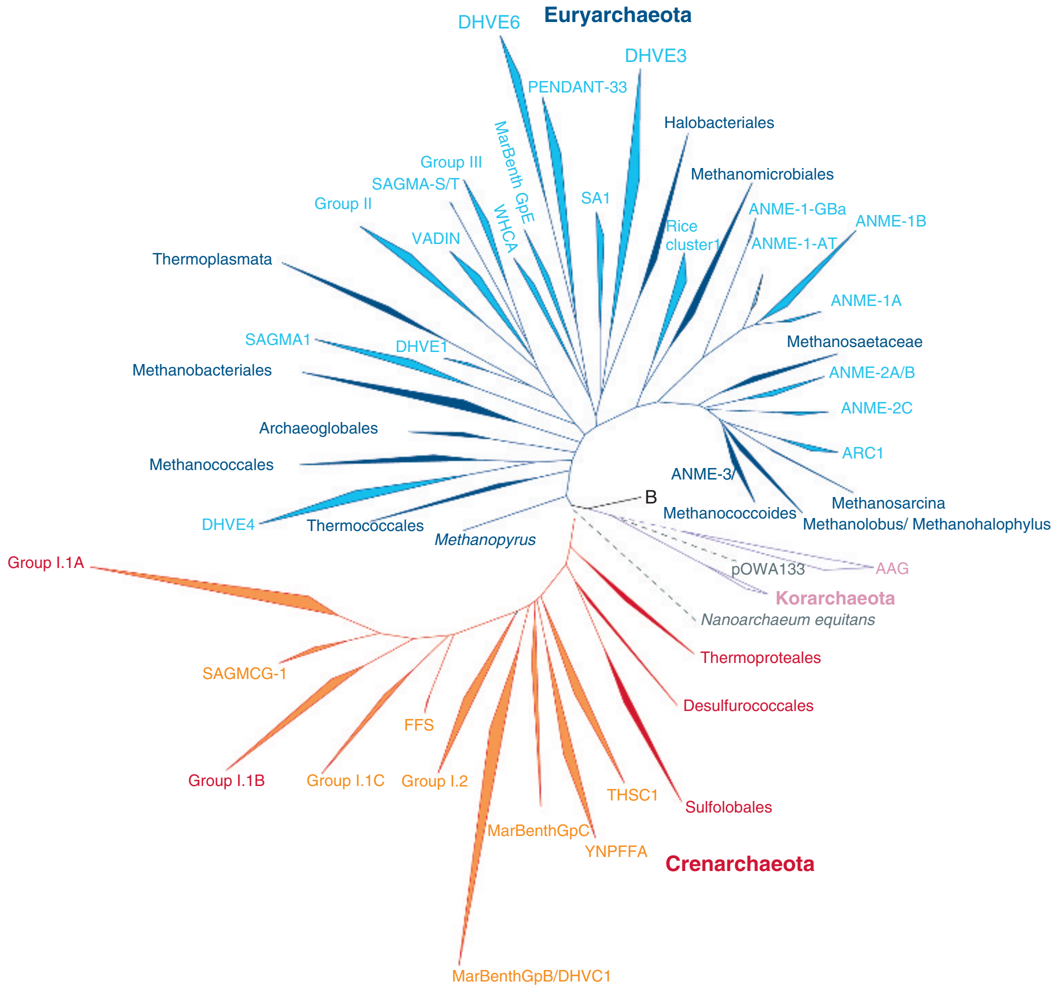
DHVE6
Euryarchaeota
DHVE3
PENDANT-33
Halobacteriales
MarBenth GpE
Group III
SAGMA-S/T
Methanomicrobiales
Group II
SA1
ANME-1-GBa
ANME-1B
WHCA
Rice cluster1
VADIN
ANME-1-AT
Thermoplasmata
ANME-1A
SAGMA1
DHVE1
Methanosaetaceae
Methanobacteriales
ANME-2A/B
ANME-2C
Archaeoglobales
ARC1
Methanococcales
ANME-3
B
Methanosarcina
Methanococcoides
Methanolobus/ Methanohalophylus
DHVE4
Thermococcales
Methanopyrus
Group I.1A
AAG
pOWA133
Korarchaeota
Nanoarchaeum equitans
Thermoproteales
SAGMCG-1
Desulfurococcales
FFS
Group I.1B
Group I.1C
Group I.2
THSC1
Sulfolobales
MarBenthGpC
YNPFFA
Crenarchaeota
MarBenthGpB/DHVC1

Figure 4 An unrooted tree of the Archaea emphasizing 16S rRNA sequences obtained from culture-independent methods. The major phyla are bolded, with Euryarchaeota in blue and Crenarchaeota in orange. 'B' indicates the position of bacterial outgroups. Named phyla and dark triangles refer to the cultured and acronyms refer to the many noncultured Archaea. Dotted lines refer to uncertainties in phylogenetic positions. Modified from Schleper C, Jurgens G, and Jonuscheit M (2005) Genomic studies of uncultivated archaea. *Nature Reviews Microbiology* 3: 479–488.

many genes interrupted by introns, are the more ancient group, evolving from a protocellular 'RNA world'. Both branches of prokaryotes are hypothesized to have evolved subsequently by loss of the nuclear membrane and introns, and additional simplification of their genomes. Therefore, a degree of uncertainty still exists with respect to how the last universal common ancestor of all life on Earth diverged to form the three separate primary lineages. Predicted characteristics of the common ancestor as a cellular organism with a cytoplasmic membrane and a sophisticated translation apparatus are however generally accepted today.

Archaeal Ecology and Environmental Biology

Traditionally, the Archaea have been recognized as microorganisms that thrive in extreme habitats (**Figure 3**) because nearly all of the originally cultivated archaeal microorganisms were found to be extremophilic (halophilic, methanogenic, or thermophilic). However, over the past 30 years a combination of culture-dependent and culture-independent techniques has shown that archaeal microorganisms are widespread, and can be detected in most common and nonextreme environments as well. In fact, detection of archaeal 16S rRNA gene sequences through sequencing of environmental samples and probing archaeal cells using fluorescent-labeled probes (fluorescent *in situ* hybridization) have led to the realization that a much greater diversity of Archaea exists than was previously known (**Figure 4**).

Global Distribution of Archaea

Although Archaea were originally discovered inhabiting extreme environments using culture-dependent techniques, their global distribution has been shown primarily through recent studies using culture-independent techniques. With over 8000 archaeal 16S rRNA gene sequences deposited in databases, it has become clear that the majority of known groups of Archaea are still not available as pure cultures, making an in-depth study of these organisms quite challenging. However, with few exceptions (i.e., Korarchaeota and Nanoarchaeota), the uncultured Archaea have all been shown to be members of the two kingdoms Crenarchaeota or Euryarchaeota (**Figure 4**).

Novel archaeal 16S rRNA sequences have been identified from a variety of microbial habitats, including fresh and salt water, hydrothermal vents and thermal springs, and diverse soil types. The importance of these Archaea has been explored through the use of 16S rRNA gene libraries and other culture-independent methods. Although these approaches are not precise tools showing a full representation of the microbial community in an environment, they can be used as a good approximation. Such studies have shown that Archaea account for up to 60% of prokaryotic cells in certain pelagic marine environments and up to 80% in some marine sediments. As a whole, it has been estimated that the Earth's oceans sustain $\sim 10^{28}$ archaeal cells. Examples of uncultured marine Archaea include members of group I.1A, group II, and marine groups I–III (see **Figure 4**). It has also been shown that certain Euryarchaeota, predominately anaerobic methane-oxidizing Archaea (ANME), comprise up to 50% of microbial mats of cold methane seeps found in the world's oceans. Archaeal cells or their 16S rRNA genes have also been detected in freshwater environments and comprise up to 5% of the prokaryotic population.

A few archaeal strains have been cultivated from hydrothermal vent communities. However, through culture-independent techniques, numerous novel strains have been identified, for example in the plethora of microenvironments that dominate vent communities. Estimates of microbial diversity are made more difficult to obtain in these environments due to their remoteness. However, scientists have predicted that Archaea do comprise a majority of cells in hydrothermal microenvironments. Typical Archaea found in these environments include marine group I, the marine benthic groups, ANME, and novel genera of *Thermococcales*, *Archaeoglobales*, and *Nanoarchaeota*. Thermal springs have also been a rich source for novel Archaea, most of which have resisted laboratory cultivation. Estimates of archaeal diversity in thermal spring environments vary in number; however, archaeal cells commonly make up a substantial fraction of the prokaryotic cell population. Novel 16S rRNA genes of terrestrial hot spring Crenarchaeota includes group 1, Sulfolobales, and Korarchaeota (**Figure 4**).

Culture-independent methods have estimated that up to 5% of the microbial community in soils are Archaea, with the Crenarchaeota alone accounting for up to 3% in some niches. Soil environments are the Earth's most diverse ecosystems and harbor a species richness that is 20-fold higher than that of the ocean. Archaea commonly found in soils include members of the group I.1B and ANME.

Attempts to bring novel noncultured archaeal microorganisms into axenic, symbiotic, or whole community culture are vigorous and ongoing. Besides using growth on traditional media, some investigators have used geological sampling data from the environment in which the organism was obtained, to try and recreate the environment in laboratory media. Others have used semipermeable chambers in which nutrients flow through but cells remain sequestered to stimulate growth. This method results in cell exposure to nearly natural nutrient environments and has been successful for the enrichment of some previously nonculturable species. A most interesting case is for the population of *Cenarchaeum symbiosum* cells in symbiotic association with the marine sponge, *Axinella mexicana*. Sufficient quantities of the DNA of this psychrophilic species were obtained for the reconstruction of a representative complete genome by metagenomic sequencing. The assembled genome sequence suggested that *C. symbiosum* may have ammonia oxidizing capability.

With the advent of extremely high-throughput metagenomic sequencing and single-cell genome-sequencing methodologies, it may be possible to perform complete genome sequencing of many more archaeal organisms and model their roles in ecology. Sampling the surrounding environment for possible primary sources of energy can help corroborate their ecological roles. This is often carried out by measuring the concentrations of molecules that are already known to serve as primary energy sources (various carbon and nitrogen compounds, hydrogen, etc.).

Halophilic Archaea

All salt-loving halophilic Archaea (also called haloarchaea) belong to the kingdom Euryarchaeota and have been classified into a single order (Halobacteriales) and family (Halobacteriaceae); however, a diverse and increasing number of genera (28 at present) have been described (**Table 1**). Haloarchaea have been isolated from numerous environments of varying salinity and generally dominate over Bacteria and a few Eucarya at the highest salinity extremes. Haloarchaea predominate in

Table 1 Taxonomy of Archaea

Euryarchaeota[a]		
Archaeoglobales	Archaeoglobaceae	*Archaeoglobus*
		Ferroglobus
		Geoglobus
Halobacteriales	Halobacteriaceae	*Haladaptatus*
		Halalkalicoccus
		Haloalcalophilium
		Haloarcula
		Halobacterium
		Halobaculum
		Halobiforma
		Halococcus
		Haloferax
		Halogeometricum
		Halomicrobium
		Halopiger
		Haloplanus
		Haloquadratum
		Halorhabdus
		Halorubrum
		Halosarcina
		Halosimplex
		Halostagnicola
		Haloterrigena
		Halovivax
		Natrialba
		Natrinema
		Natronobacterium
		Natronococcus
		Natronolimnobius
		Natronomonas
		Natronorubrum
Methanobacteriales	Methanobacteriaceae	*Methanobacterium*
		Methanobrevibacter
		Methanosphaera
		Methanothermobacter
	Methanothermaceae	*Methanothermus*
Methanococcales	Methanocaldococcaceae	*Methanocaldococcus*
		Methanotorris
	Methanococcaceae	*Methanococcus*
		Methanothermococcus
Methanomicrobiales	Methanocorpusculaceae	*Methanocorpusculum*
	Methanomicrobiaceae	*Methanoculleus*
		Methanofollis
		Methanogenium
		Methanolacinia
		Methanomicrobium
		Methanoplanus

(*Continued*)

Table 1 (Continued)

	Methanospirillaceae	*Methanospirillum*
	Unclassified	*Methanoregula*
		Methanocalculus
		Methanolinea
Methanopyrales	Methanopyraceae	*Methanopyrus*
Methanosarcinales	Methanosaetaceae	*Methanosaeta*
		Methanotrix
	Methanosarcinaceae	*Methanimicrococcus*
		Methanococcoides
		Methanohalobium
		Methanohalophilus
		Methanolobus
		Methanomethylovorans
		Methanosalsum
		Methanosarcina
Thermococcales	Thermococcaceae	*Palaeococcus*
		Pyrococcus
		Thermococcus
Thermoplasmatales	Ferroplasmaceae	*Ferroplasma*
	Picrophilaceae	*Picrophilus*
	Thermoplasmataceae	*Thermoplasma*
	Unclassified	*Thermogymnomonas*
Unclassified		*Aciduliprofundum*
		Methanosphaerula
Crenarchaeota[a]		
Caldisphaerales	Caldisphaeraceae	*Caldisphaera*
Cenarchaeales	Cenarchaeaceae	*Cenarchaeum*
Desulfurococcales	Desulfurococcaceae	*Acidilobus*
		Acidococcus
		Aeropyrum
		Desulfurococcus
		Fervidococcus
		Ignicoccus
		Staphylothermus
		Stetteria
		Sulfophobococcus
		Thermodiscus
		Thermofermentum
		Thermosphaera
	Pyrodictiaceae	*Geogemma*
		Hyperthermus
		Pyrodictium
		Pyrolobus
	Unclassified	*Caldococcus*
		Ignisphaera
Nitrosopumilales	Nitrosopumilaceae	*Nitrosopumilus*
Sulfolobales	Sulfolobaceae	*Acidianus*
		Metallosphaera
		Stygiolobus
		Sulfolobus
		Sulfurisphaera
		Sulfurococcus
Thermoproteales	Thermofilaceae	*Thermofilum*
	Thermoproteaceae	*Caldivirga*
		Pyrobaculum
		Thermocladium
		Thermoproteus
		Vulcanisaeta
Nanoarchaeota[a]		
		Nanoarchaeum
Korarchaeota[a]		*Korarchaeum*

[a] Major phyla (kingdoms) are listed in bold, with subphyla following. First column contains orders; second column, families; and third column, genera.

environments such as artificial crystallizer ponds, shallow ponds for isolating salts from the sea, as well as natural solar salterns, where isolates of *Halobacterium*, *Halorubrum*, *Haloarcula*, *Halogeometricum*, and *Haloquadratum* (including a square-shaped species) are typically detected. The microbial composition of the Dead Sea, which contains an unusually high concentration of magnesium, and ancient salt deposits, some as old as 200 million years (from the Permian period), have yielded haloarchaeal isolates, such as *Haloarcula*, *Halobacterium*, *Halococcus*, *Haloferax*, and *Halorubrum*. The true age of isolates from ancient salt deposits is quite controversial, since some metabolic activity occurring in the entrapped state cannot be strictly ruled out. Another typical environmental niche for haloarchaea are other neutral and alkaline hypersaline lakes, for example, the north arm of Great Salt Lake in the western United States (separated from the south arm by a railroad causeway), Lake Assal in Djibouti, and Lake Magadi in the Rift Valley of Africa, where species of *Haloarcula*, *Natronococcus*, and *Natronomonas* have been isolated. Species of *Halobiforma*, *Halomicrobium*, *Halogeometricum*, and *Haloterrigena* have been isolated from less salty environments such as coastal oceans, marshes, and soils. Traditionally, halophilic Archaea, such as *Halobacterium*, were isolated from salted protein sources such as fish sauces and animal hides.

Haloarchaea are able to perform aerobic and anaerobic respiration; however, most are facultative aerobes (**Figure 5**). All genera of haloarchaea are able to use amino or organic acids as a carbon source. Other carbon sources utilized by haloarchaea include sugars, glycerol, and hydrocarbons. Since their natural environments often have low oxygen concentrations (oxygen solubility is reduced by high salinity), many haloarchaea are able to grow anaerobically. Terminal electron acceptors during anaerobic growth include dimethylsulfoxide, trimethylamine, fumarate, nitrogen oxide, and in some cases nitrate. Certain species of haloarchaea are able to grow anaerobically via the fermentation of arginine.

Some haloarchaea, for example, *Haloarcula*, *Halobacterium*, and *Halorubrum*, produce a light-driven proton pump, bacteriorhodopsin, in their cell membrane (**Figure 5**). In some strains, high levels of bacteriorhodopsin produced in response to limiting oxygen and high light intensity form a two-dimensional crystalline lattice called the purple membrane. The purple color is due to light absorption by the chromophore retinal that is chemically linked to the protein bacterio-opsin, which is similar to the photopigment in the visual systems of higher Eucarya. Under conditions with sufficiently high levels of bacteriorhodopsin and light intensity, cells may use the proton-motive force generated to grow phototrophically for a period of time. Retinal proteins similar to bacteriorhodopsin in *Halobacterium*, known as sensory rhodopsins, can also mediate phototactic responses, swimming toward beneficial green light and away from damaging blue and UV light. A third class of retinal protein, halorhodopsin, in *Halobacterium* acts as a light-driven chloride pump (**Figure 5**).

Haloarchaea have been shown to resist the denaturing effects of high salt concentrations through a process of selective uptake of salts known as ‘salting in’, which is used by few nonarchaeal organisms. The accumulation of salts internally, mainly KCl, reduces osmotic stress to the

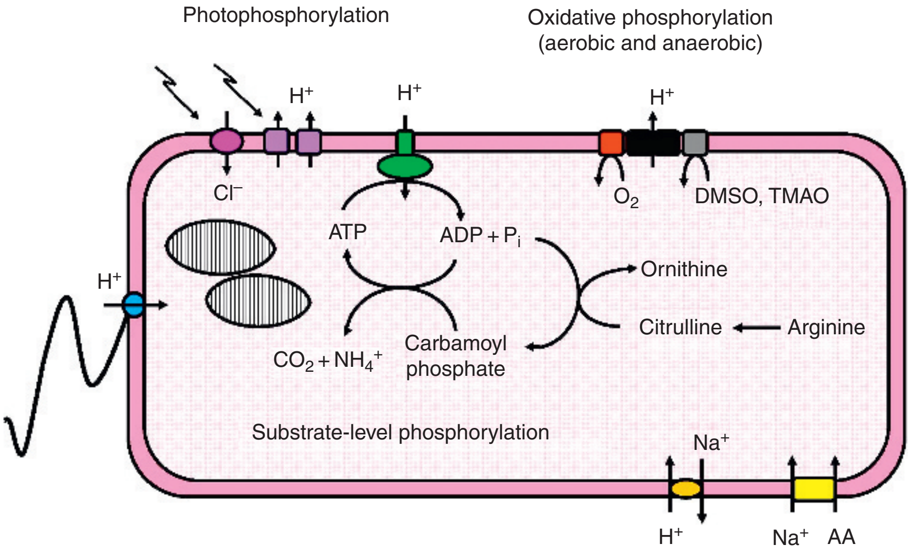

Figure 5 Physiology of haloarchaea. Haloarchaea have the capability to grow by aerobic or anaerobic respiration (oxidative phosphorylation), anaerobic fermentation (substrate-level phosphorylation), and/or photophosphorylation (using light-driven pumps in the membrane and lemon-shaped gas vesicles for flotation). The membrane potential and proton-motive force are also used to drive many metabolic processes, such as sodium ion extrusion, amino acid uptake, and flagellar (extracellular line) rotation.

cell membrane but creates an intracellular milieu that is harsh and challenging for biological macromolecules. The internal salt concentration of most halophilic species, like *Halobacterium*, has been measured to be as high as the natural environment, up to 5 M salts, which would result in desolvation, aggregation, denaturation, and precipitation (via salting out) of most nonhaloarchaeal proteins. Some DNA sequences, for example, alternating GC sequences, morph into a left-handed form, called Z-DNA. Haloarchaeal cells maintain high internal KCl concentration and relatively low NaCl concentration, via both membrane potential and ATP-driven potassium uptake systems and sodium–proton antiporters (**Figure 5**). The sodium-motive force is important for metabolic activities, like the uptake of amino acids, which generally are present in high concentrations during periods of increased salinity due to evaporation and resulting decline and decomposition of less halophilic species. Genomic analysis has also shown that the proteins of haloarchaea are highly acidic, and structural studies have revealed that surface negative charges facilitate the formation of a hydration shell, increasing their solubility and decreasing aggregation and precipitation. The high solar illumination of many hypersaline environments has also resulted in development of tolerance to radiation for haloarchaea via active DNA repair mechanisms, including both light repair (photolyase) and dark repair (nucleotide excision repair) systems. In fact, the most radiation-resistant strain as well as the most space condition-tolerant vegetative cells to have been found thus far are both haloarchaea.

Methanogenic Archaea

Methanogenic Archaea are microorganisms that are capable of producing methane gas, a potential fuel source as well as a greenhouse gas that has been implicated in global warming. Taxonomically, all methanogens are members of the kingdom Euryarchaeota, like the haloarchaea, but they form a broad group comprising 5 orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, and Methanosarcinales), 10 families (Methanobacteriaceae, Methanocaldococcaceae, Methanococcaceae, Methanocorpusculaceae, Methanomicrobiaceae, Methanopyraceae, Methanosaetaceae, Methanosarcinaceae, Methanospirillaceae, and Methanothermaceae) and 31 genera (**Table 1**). These organisms are extremely sensitive to oxygen and therefore selectively inhabit strictly anoxic environments. Methanogens serve an important role in the global carbon cycle, completing the conversion of organic carbon into methane gas. This process is syntropic, meaning that the products of metabolic activities of other microorganisms are used as the substrates for methanogenesis.

The substrates used to generate methane can be arranged into three groups: methyl or other one-carbon type (methylotrophic), carbon dioxide type (hydrogenotrophic), and acetoclastic (**Figure 6**), and methanogens have been found in three main types of ecosystems. The most common methanogenic environments are freshwater sediments, swamps, peat bogs, rice fields, and sewage digesters, where typical methanogens include species of *Methanobacterium*, *Methanosarcina*, and *Methanosaeta.* In these environments, communities of resident bacteria degrade biopolymers into alcohols and fatty acids, and the fatty acids subsequently into acetate and carbon dioxide. Some bacteria in these communities also produce hydrogen as a metabolic end product. Methanogens then utilize these products to generate methane. The most widely used substrate for methanogenesis is acetate, which accounts for two-thirds of the methane generated in these environments.

Another type of methanogenic environment exists inside multicellular organisms (i.e., rumen fluid and digestive tracts) where species of *Methanobacterium*, *Methanobrevibacter*, and *Methanimicrococcus* are common. Unlike the external environments, the digestive systems of animals actively absorb intermediates from the breakdown of complex organics produced by bacteria and therefore these nutrients are available only for a relatively short time. As a result, the hydrogenotrophic methanogens grow faster and predominate over those that utilize acetate in these environments.

In the third type of environment, the substrates for methanogenesis are not of biological but geological origin. In these environments, geothermal gases (e.g., carbon dioxide and hydrogen gas) are the substrates used by methanogens. These environments are often also hot (e.g., geysers, solfataras, and hot springs) and species of *Methanothermus*, *Methanothermobacter*, and many other diverse species have been isolated (**Table 1**).

The main adaptive strategy for methanogens in the environment is to exist as part of syntropic communities or consortia with Bacteria. Due to the extreme sensitivity of methanogens to oxygen, they inhabit the most reducing zones within the various habitats, for example, the strictly anaerobic zone of microbial mats in sediment. Within the anoxic zones, methanogens may dominate or compete with other anaerobic Bacteria, such as sulfate-reducing Bacteria for hydrogen. Hydrogenotrophic methanogens are usually less competitive in sulfate-rich marine environments since sulfate reducers are able to outcompete them for hydrogen. However, even in these environments, methanogens may still utilize one-carbon substrates, such as methanol, methylamines, and methionine, which are not used by the sulfate-reducing Bacteria.

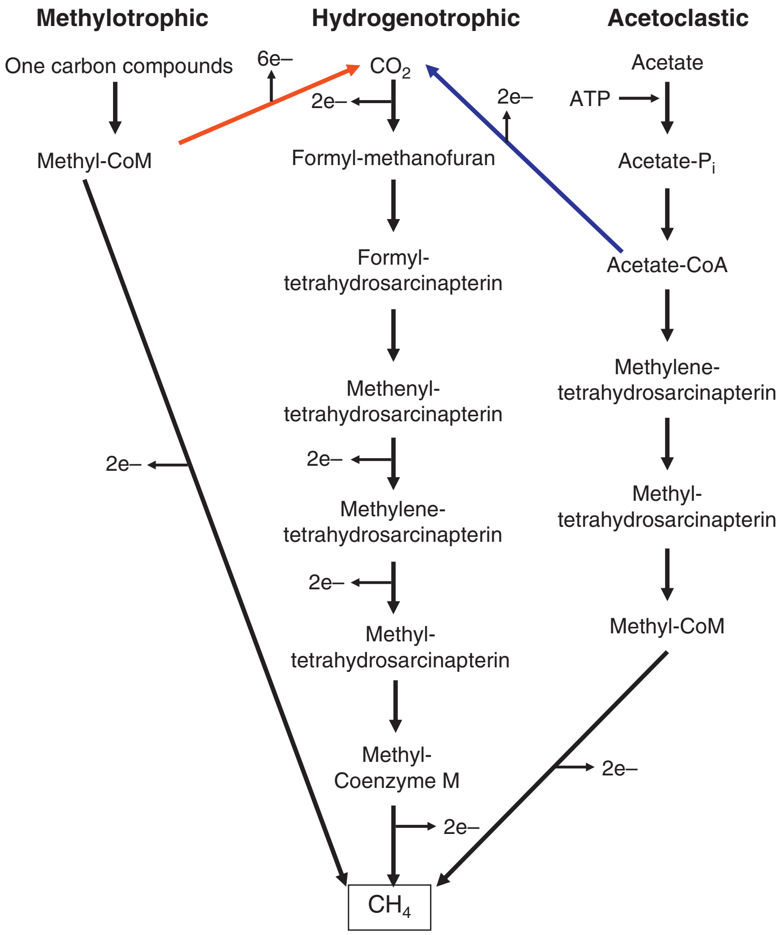
Methylotrophic
Hydrogenotrophic
Acetoclastic
One carbon compounds
6e–
CO2
2e–
2e–
Acetate
ATP
Methyl-CoM
Formyl-methanofuran
Acetate-Pi
Formyl-tetrahydrosarcinapterin
Acetate-CoA
Methenyl-tetrahydrosarcinapterin
Methylene-tetrahydrosarcinapterin
2e–
2e–
Methylene-tetrahydrosarcinapterin
Methyl-tetrahydrosarcinapterin
2e–
Methyl-tetrahydrosarcinapterin
Methyl-CoM
Methyl-Coenzyme M
2e–
2e–
CH4

Figure 6 Pathways of methanogenesis. Overview of the three pathways responsible for the biogenesis of methane. The red arrow indicates the ability of some Archaea to reverse the hydrogenotrophic pathway to produce CO_2, which can then move in the forward direction to produce methane. The blue arrow indicates that CO, which is later converted into CO_2, is a by-product of the acetoclastic pathway.

Thermophilic Archaea

The most diverse group of cultured Archaea are thermophilic, members of which occupy both the Euryarchaeota and Crenarchaeota kingdoms. Isolates of the former kingdom consist of 3 orders (Archaeoglobales, Thermococcales, and Thermoplasmatales), 5 families (Archaeoglobaceae, Ferroplasmaceae, Picophilaceae, Thermococcaceae, and Thermoplasmataceae), and 10 genera (**Table 1**), while those of the latter kingdom consist of 5 orders (Caldisphaerales, Cenachaeales, Desulfurococcales, Sulfolobales, and Thermoproteales), 7 families (Caldisphaeraceae, Cenachaeaceae, Desulfurococcaceae, Pyrodictiaceae, Sulfolobaceae, Thermofilaceae, and Thermoproteaceae), and 32 genera (**Table 1**). These microorganisms have been isolated from solfataras, hot springs, and hydrothermal vents. *Archaeoglobus*, *Desulfurococcus*, *Pyrobaculum*, *Sulfolobus*, *Thermofilum*, and *Thermoproteus* have been found in solfataric fields and hot springs from sites in Iceland, Italy, Japan, New Zealand, Russia, and the United States. Hydrothermal vent communities can be divided into two groups: shallow and deep. Shallow vents are sites of hydrothermal activity within a few 100 m below the ocean surface. In these sites, genera such as *Palaeococcus*, *Pyrobaculum*, *Pyrococcus*, *Pyrodictium*, and *Stetteria* have been isolated. Deep vent communities are found near subsurface volcanoes and at the boundary between seawater and magma, usually kilometers beneath the ocean surface. These communities often contain *Archaeoglobus*, *Geoglobus*, *Ignicoccus*, and *Nanoarchaeum*, the last of which has been proposed to constitute a new archaeal kingdom.

Although many species of thermophiles and hyperthermophiles have been isolated, data about their metabolic capabilities and habitats are still accumulating (**Table 2**). These organisms are capable of using a wide diversity of molecules as electron donors (sulfur, iron (II), hydrogen gas, and lactate) and acceptors (sulfate, nitrate, iron (III), and oxygen). However, most thermophilic

Table 2 Thermophilic metabolic reactions

Metabolic reaction	*Organism*
Organic compounds $+ O_2 \rightarrow H_2O + CO_2$	*Sulfolobus*, *Aeropyrum*
$H_2 + ½O_2 \rightarrow H_2O$	*Sulfolobus*, *Pyrobaculum*, *Acidianus*, *Metallosphaera*, *Pyrolobus*
Organic compounds $+ H_2SO_4 \rightarrow H_2S + CO_2$	*Archaeoglobus*
Organic compounds $+ S^0 \rightarrow H_2S + CO_2$	*Thermoproteus*, *Thermococcus*, *Desulfurococcus*, *Thermofilum*, *Pyrococcus*
Organic compounds $\rightarrow CO_2 +$ fatty acids	*Staphylothermus*, *Thermoplasma*
Organic compounds $\rightarrow CO_2 + H_2$	*Pyrococcus*
Aromatic compound $+$ Fe(III) $\rightarrow HCO_3 +$ Fe(II) $+ H_2O + H^+$	*Ferroglobus*
$H_2 + S^0 \rightarrow H_2S$	*Pyrodictium*, *Thermoproteus*, *Pyrobaculum*, *Acidianus*, *Stygiolobus*, *Desulfurococcus*, *Ignicoccus*, *Stetteria*, *Sulfurisphaera*, *Thermodiscus*, *Thermofilum*
$4H_2 + SO_4^{2-} \rightarrow H_2S + 4H_2O$	*Archaeoglobus*
$H_2 + HNO_3 \rightarrow HNO_2 + H_2O$	*Pyrobaculum*, *Pyrolobus*, *Ferroglobus*
$4H_2 + HNO_3 \rightarrow NH_4OH + 2H_2O$	*Pyrolobus*
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	*Methanopyrus*, *Methanothermus*, *Methanococcus*, *Methanocaldococcus*
$6H_2O + NO_3^- + 2FeCO_3 \rightarrow NO_2^- + H_2O + 2\ Fe(OH)_3 + HCO_3^-$	*Ferroglobus*
$2S^0 + 3O_2 \rightarrow H_2S + CO_2$	*Sulfolobus*, *Acidianus*
$2S^0 + 3O_2 + H_2O \rightarrow 2H_2SO_4$	*Acidianus*, *Metallosphaera*, *Sulfolobus*
$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4$	*Acidianus*, *Metallosphaera*, *Sulfolobus*
Acetate $+ 8$Fe(III) $+ 4H_2O \rightarrow 2HCO_3^- + 8$Fe(II) $+ 9H^+$	*Ferroglobus*, *Geoglobus*
$H_2 + 6FeO(OH) \rightarrow 2Fe_3O_4 + 5H_2O$	*Pyrobaculum*
Arsenate $+ H_2 \rightarrow$ arsenite $+ H_2O$	*Pyrobaculum*

environments have very low concentrations of oxygen, and hyperthermophilic habitats (found at depths of several kilometers) are completely devoid of sunlight. Not surprisingly, a large majority of thermophilic isolates are anaerobes and all hyperthermophiles and many thermophiles are chemoautotrophic. The upper limit for hyperthermophilic life has been extended in recent years to increasingly higher temperatures (current maximum 121 °C) under high pressures (>120 MPa).

The extreme temperatures at which thermophilic and especially hyperthermophilic Archaea thrive require the adaptation of proteins, lipids, DNA, and other cellular components to prevent denaturation and degradation. These microorganisms have incorporated subtle changes in amino acid sequences that are important for stabilizing thermophilic proteins. They have a greater content of charged residues and intrahelical charge pairs forming salt bridges than mesophiles. Thermophilic proteins also appear to be smaller, and in some cases more basic, which may also result in increased stability. However, it is possible that these differences may reflect evolutionary relationships rather than stability factors. Another method used to improve the stability of proteins of thermophiles is through the action of chaperones, which help to refold denatured proteins. Thermophilic Archaea contain three families of heat shock proteins, Hsp70, Hsp60 (or thermosome family), and small heat shock proteins (sHsp). They also contain prefoldin, which assists in refolding by delivering unfolded proteins to chaperones.

Thermophiles have improved membrane lipid stability by increasing the number of saturated fatty acids in their bilayer. Hyperthermophiles also use novel dibiphytanyl glycerol tetraether components, which results in the formation of a lipid monolayer (**Figure 2**). As temperatures increase to levels experienced by hyperthermophiles, lipid bilayers may become unstable, but monolayer membranes retain stability. In order to increase the stability of DNA, hyperthermophilic microorganisms possess a novel enzyme, reverse gyrase, which introduces positive DNA supercoils and protects it against thermal denaturation. Another means of stabilizing DNA is the employment of DNA-binding proteins and compaction of the genome into chromatin. Some thermophiles have a high internal concentration of potassium ions, like haloarchaea, which may help prevent chemical damage that can occur at high temperatures.

Other Extremophilic Archaea

Psychrophilic Archaea

Over 80% of the Earth's biosphere is at or below 4 °C and harbors a wide variety of species, bacterial, archaeal, and eucaryal, capable of growth at low temperatures. Relatively little is known about most psychrophilic organisms that grow and thrive in these environments, and compared to the thermophilic Archaea, there are very few well-characterized psychrophilic Archaea. Only three cultured species of psychrophilic Archaea – *Methanococcoides burtonii*, *Methanogenium frigidum*, and *Halorubrum lacusprofundi* – have been studied in detail, all of which were isolated from Antarctic lakes (**Figure 3**). In addition, *C. symbiosum*, though not yet axenically cultured, has been maintained in the laboratory and studied through cocultivation with its marine sponge host.

Like thermophiles, psychrophilic Archaea have also adapted their proteins, lipids, and other biomolecules for activity at an extreme temperature (i.e., low temperature). There is still uncertainty on the precise mechanisms of adaptation of psychrophilic proteins, although the present view is that multiple subtle changes in overall protein structure are responsible for cold activity. Psychrophiles also employ 'antifreeze' proteins that inhibit formation of ice crystals within the cell to mitigate their damaging effects. Lipids of psychrophiles also incorporate more unsaturation allowing them to remain fluid at lower temperatures.

Acidophilic and alkaliphilic Archaea

Acidophilic and alkaliphilic microorganisms, which include diverse species representing all three domains of life, thrive at the extremes of pH. Acidic environments have been studied all around the world and consist of natural (solfataras, acidic springs) as well as man-made sites (acid mine drainage, bioleaching reactors). Typical genera of the acidophilic Archaea include *Sulfolobus*, *Ferroplasma*, and *Thermoplasma*. Alkaline environments also consist of natural (e.g., soda lakes) and man-made sites. Soda lakes have stable pH values at or above 10. The evaporitic conditions at these lakes lead to high concentrations of sodium carbonate and usually other salts, such as sodium chloride. Therefore, haloarchaea, such as *Haloarcula*, *Natronococcus*, and *Natronomonas* are typical microorganisms in these environments. It was thought that alkaliphiles could not survive at elevated temperatures. However, recently several alkaliphilic thermophile species (e.g., *Thermococcus*) have been isolated, such as on Vulcano Island, Italy. Methanogenic alkaliphiles (e.g., *Methanosalsum*) have also been isolated.

How acidophilic and alkaliphilic Archaea are able to thrive in pH extremes while keeping an internal pH close to neutral is not well understood. Studies have shown that the membrane of some acidophiles have an extremely low proton permeability at acidic pH (<4.0), while at neutral pH they are unable to assemble into liposomal structures. This suggests that loss of membrane integrity may be the reason why these organisms are able to grow preferentially at low pH. For alkaliphiles, modifications to their membrane and cell wall are also important. These organisms have developed two levels of defense from the external high-pH environment. The first is to have acidic polymers in their cell wall. It has been suggested that the negative charges in the cell wall repel hydroxide ions in the external alkaline environment. The second defense is by the combined action of the sodium/proton antiporter system, potassium uptake, and ATPase-driven proton expulsion, which help to maintain physiological pH internally.

Archaeal Viruses

Since the initial discovery of archaeal viruses with head–tail phenotype, many new archaeal viruses with novel and unique shapes (fusiform, bottle-shaped, droplet-shaped, linear, and spherical) have been found. The number and diversity of these novel forms indicate that they predominate in the environment. The genes of sequenced archaeal viruses, except for the head–tail variety, do not show significant homology to nonarchaeal varieties or to proteins of recognizable function. All cultured archaeal viruses have double-stranded DNA; however, few genes are shared between archaeal gene families though there is some evidence of sharing within families. Studies of archaeal viruses have also led to a hypothesis that DNA replication may have evolved independently in multiple domains.

Novel Molecular and Genetic Characteristics of Archaea

The process of information transfer (known as the central dogma of molecular biology, with information flowing from DNA → RNA → protein) is the same in all the three domains; however, in Archaea these processes are more closely related to those of Eucarya than they are to those of Bacteria. These findings led to comparative analysis of many of the highly conserved macromolecular species involved in DNA replication, transcription, and translation. In addition, bacterial aspects of the molecular biology of Archaea were also noted, including the absence of a nuclear membrane and coupling of transcription and translation. Interestingly, the translation apparatus of some methanogenic species were also found to be capable of incorporating nonstandard amino acids (e.g., selenocysteine and pyrrolysine), and the proteins of halophiles and some thermophiles were found to be highly charged with an abundance of acidic and basic amino acids, respectively.

Much of the current knowledge of archaeal molecular biology is based on genome sequencing and bioinformatic approaches. In addition, a few systems have been studied in depth using genetic and biochemical analysis. For example, biochemical studies of transcription and DNA replication have been conducted in detail in certain methanogenic and thermophilic Archaea (e.g., *Methanococcus thermolithotrophicus*, *Pyrococcus furiosus*, and *Sulfolobus shibatae*). Other methanogens, haloarchaea, and thermophiles (e.g., *Methanococcus maripaludis* and *Methanosarcina acetivorans*, *Halobacterium* sp. NRC-1 and *Haloferax volcanii*, and *Sulfolobus solfataricus* and *Thermococcus kodakarensis*) have also provided genetic systems for the study of fundamental processes through gene knockout and replacement systems. In many cases, specialized cloning and expression vectors and novel selectable markers (e.g., mevinolin, simvastatin, puromycin, and neomycin-resistance) were developed to aid in analysis.

Genomic Architecture

The genomes of Archaea are similar in size and structure to those in Bacteria, with circular chromosomes 0.5–5.75 Mbp in size requiring significant (1000-fold) compaction for packaging into the small prokaryotic cells. Thermophilic and especially hyperthermophilic species also require the genome to be protected from thermal denaturation. Archaea have evolved chromatin proteins to maintain reversible compaction, which is essential for genomic function in DNA replication and transcription. The archaeal chromatin proteins are of two main types, histones similar to the ubiquitous eucaryal proteins, and Alba proteins, which are uniquely archaeal. In addition, several other families of chromatin proteins have been found in Archaea, but are more specific to certain phyla.

Histone proteins are mainly found in the Euryarchaeota and in a few early branching Crenarchaeota (e.g., *C. symbiosum*). These organisms encode 1–6 histone proteins in their genomes, which dimerize for stability, and form tetramers and sometimes hexamers in the presence of DNA. This is in contrast to the well-conserved four histone proteins in Eucarya that form octomers and nucleosomes in association with DNA. The difference between archaeal and eucaryal histones may reflect the need for prokaryotic archaeal cells to access many regions of their genome simultaneously as opposed to differentiated eucaryal cells that require access to more limited regions of their genomes. Alba proteins are abundant in Crenarchaeota and some thermophilic and hyperthermophilic Euryarchaeota, and the corresponding genes are present in one or two copies within most thermophilic archaeal genomes. Alba dimerizes for stability and binds to DNA and possibly RNA. Alba is subject to acetylation, which decreases its affinity for DNA binding.

Among hyperthermophilic Archaea (as well as hyperthermophilic Bacteria), the thermal denaturation of genomic DNA is inhibited by the introduction of positive supercoils using the reverse gyrase enzyme. Reverse gyrase is a multidomain protein, with an N-terminal portion containing a helicase domain and a C-terminal portion consisting of a type IA topoisomerase domain. It adds positive supercoils by a combination of actions of the two domains. This novel topoisomerase increases the rigidity of DNA and stabilizes double-stranded DNA at high temperatures. Reverse gyrase has also been shown to bind to internal nicks as well as the ends of DNA, stabilizing them by reducing the rate of strand breakage.

DNA Replication

DNA replication in Archaea contains elements of both the bacterial and eucaryal systems, with eucarya-like replication proteins acting to replicate a bacteria-like genome. Archaea initiate replication at one (e.g., *Pyrococcus abyssi*) or multiple sites (*S. solfataricus*) in their circular chromosome. These sites – found in intergenic regions – contain large sequence repeats, which are typically inverted, and an AT-rich region called the duplex unwinding element. These chromosomal DNA replication origins (origin binding or ORB) are usually but not always near an Orc/Cdc6 gene and serve as a binding site for the resulting protein. Through recently solved cocrystal structure analysis, the eucarya-like *Aeropyrum pernix* and *S. solfataricus* Orc/Cdc6 proteins were found to bind the DNA replication site via their winged helix domain, inserting the recognition helix into the major groove and wing into the minor groove (**Figure 7**). The AAA$^+$ domain also contacts the minor groove, which results in unwinding and kinking, by up to 35°, of the DNA. This local opening of the DNA helix leads to recruitment of the minichromosome maintenance (MCM) complex, as in Eucarya. The MCM complex continues the unwinding of DNA and interacts with another protein complex, GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese), which links MCM with the archaeal primase. Once the RNA primers are created, the sliding clamp loader replication factor complex opens and then loads the sliding clamp, or, proliferating cell nuclear antigen (PCNA), onto the DNA. PCNA tethers the DNA polymerase to DNA and increases the length of DNA chains produced. PCNA has also been shown to interact with the flap endonuclease – both of which are eucarya-type proteins – as well as DNA ligase I and uracil DNA glycosylase, all of which are involved in DNA replication and/or repair in Archaea. While most studies of DNA replication have been conducted using biochemical approaches using both Crenarchaeota and Euryarchaeota, the essential nature of the genes for most of these replication factors have been demonstrated using the genetic system of the halophilic Euryarchaeote, *Halobacterium* sp. NRC-1.

There are two classes of DNA polymerase involved in DNA replication in Archaea: PolB, similar to the eucaryal PolB family enzymes, and PolD, a Euryarchaeota-specific PolD family enzyme. The Crenarchaeota possess only PolB-type DNA polymerases, while the Euryarchaeota contain essential PolB and PolD family polymerases. However, the crenarchaeote, *C. symbiosum*, is an exception to this rule. The PolB polymerases are DNA primer-directed DNA polymerases that do not require PCNA for efficient synthesis but do require it for strand displacement. The PolD polymerases prefer a primed template for DNA binding and extension, and while they do not require PCNA for strand displacement, they do require it

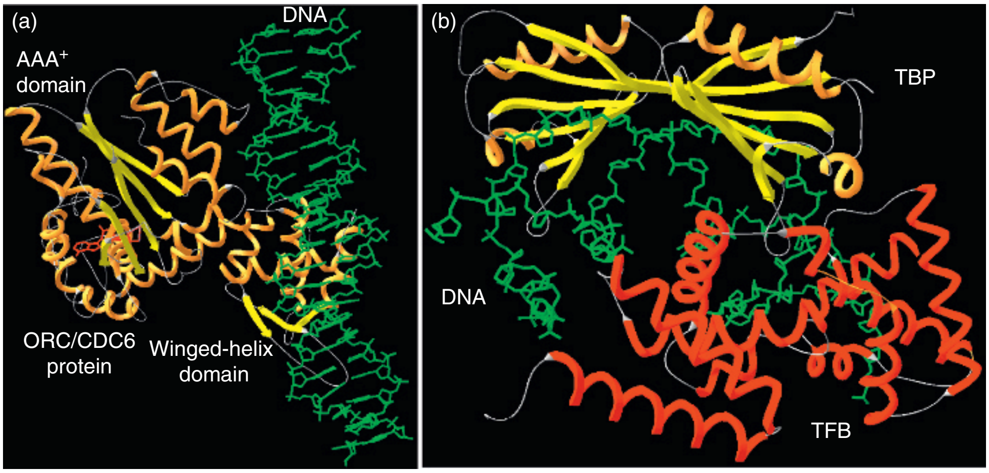

Figure 7 Structure of DNA replication and transcription initiation proteins bound to DNA. (a) Model structure of an Orc/Cdc6 protein bound to an origin DNA. DNA strands are green. Cdc/Orc6 protein is colored according to secondary structure (α-helices are orange and β-sheets yellow). ADP and Mg^{2+} in the structure are colored pink respectively. (b) Model structure of the TBP–TFB–DNA complex. Green strands correspond to the transcriptional promoter DNA.

for efficient DNA synthesis. It has been hypothesized that in the Euryarchaeota, PolB acts as the leading strand DNA polymerase, while PolD acts as the lagging strand DNA polymerase. In the Crenarchaeota, PolB family polymerases may act on either side of the replication fork (leading or lagging strand). The RNA primers synthesized by the archaeal primase are usually removed by a type II RNaseH; however, some diverse Archaea, such as *Halobacterium* sp. NRC-1, *Sulfolobus tokodaii*, and *Pyrobaculum aerophilum*, encode a type I RNaseH as well. In such organisms, the type II RNaseH and flap endonuclease act cooperatively to process Okazaki fragments on the lagging strand, while type I RNaseH removes the last ribonucleotide of the RNA primer of the Okazaki fragment. Once the primers are removed, eucarya-type single-strand binding (replication protein A) proteins bind the DNA and DNA ligases (either ATP- or NAD^+-dependent), and seal nicks in the DNA.

DNA Repair

Archaea have DNA repair proteins (like their replication counterparts) that are usually more similar to their eucaryal equivalents. An important property of archaeal replicative B family DNA polymerases (PolB family) is a 'read ahead' function for uracil, which stalls the polymerase when the nucleotide is encountered in the template DNA. This type of repair is most important for the thermophilic Archaea since uracil resulting from cytosine deamination is more frequent at high temperatures. Archaeal mismatch repair machinery differs from the common bacterial MutS/MutL pathway, although the low mutation frequency in some species suggests the existence of an efficient mechanism. For repair of remaining unrepaired DNA lesions, some Archaea encode a lesion bypass DNA polymerase, for example, Dpo4 in *S. solfataricus*. Dpo4 (a DinB homologue in Bacteria) is a member of the Y family of error-prone polymerases, and can bypass UV photoproducts, such as cyclobutane pyrimidine dimers and abasic sites. The distribution of translesion bypass polymerases in Archaea indicates that they are generally found in organisms exposed to UV radiation such as the Sulfolobales and Halobacteriales. Many species with Y family polymerases also contain the UV photoproduct repair enzyme photolyase. Methanosarcinales also contain these enzymes, suggesting that these strictly anaerobic Archaea are also exposed to UV radiation under some circumstances. Deep-sea species have no exposure to UV or visible light, and therefore lack these enzymes.

The bacterial excision repair machinery (UvrABC) is present in certain mesophilic Euryarchaeota, and has been shown to be genetically responsible for nucleotide excision repair in at least one species, *Halobacterium* sp. NRC-1. Thermophilic Archaea likely use a different pathway for nucleotide excision repair, possibly homologues of the eucaryal enzymes, XPF-ERCC1 and Fen-1 (a homologue of XPG) nucleases, and XPB and XPD helicases. However, not all Archaea contain all of these repair proteins and others, such as the damage recognition proteins XPA and XPC found in Eucarya, are absent altogether. Moreover, some of these enzymes are involved in other cellular processes, for example, Fen1 is involved in Okazaki fragment processing in DNA replication, and XPB and XPD are involved in transcription initiation.

Transcription

The transcriptional machinery in Archaea also has distinctly eucaryal features, although their genes are frequently organized into transcription units or operons, as found in Bacteria. Transcription in Archaea is carried out using a simplified version of the eucaryal RNA polymerase II-like system, enzymes with 11 or 12 subunits, compared to only 4 subunits in the single bacteria-type RNA polymerase. The RNA polymerase of *Pyrococcus* and the Crenarchaeota have 11 subunits (RpoBA′A′′DE′FLHNKP), while methanogens and haloarchaea have 12 subunits, with the B subunit split into B′ and B″. Crystal structures of RNA polymerase from both Bacteria and Eucarya (*Escherichia coli* and *Saccharomyces cerevisiae*, respectively) have been compared to the subunits of the *P. furiosus* enzyme to identify common structural motifs.

The promoter structure and transcription initiation factors of Archaea are also eucaryal in nature. Archaeal promoters have an AT-rich region (TATA box) located about 25 bp upstream of the transcription start point, like most eucaryal RNA polymerase II promoters. This is distinct from the bipartite bacterial promoter, which possesses −10 and −35 recognition elements. Archaea also use the eucarya-like TATA-binding protein (TBP) and transcription factor IIB (TFB) (**Figure 7**), although not the host of other eucaryal transcription initiation factors, or σ factors, found in Bacteria. TBP binds to the TATA box upstream of the transcriptional start site, while the TFB protein binds a B-recognition element immediately upstream of the TATA box. Interestingly, both the TBP and TFB proteins contain two imperfectly repeated sequences from internal gene duplications. The genomes of most archaeal species sequenced to date have shown just one or two copies of the corresponding genes. However, multiplicity of transcription factor genes is common in some Archaea, for example, the haloarchaea, where up to six TBP genes and nine TFB genes have been found in the genome. Genetic analysis in haloarchaea showed that although a fraction of these genes may be deleted, multiple transcription factor genes are essential. Since no TATA-interacting proteins have been found in the Archaea, haloarchaea likely contain a novel regulatory system involving recognition of different promoters by specific TBP–TFB combinations (e.g., for their response to heat shock). Consistent with this hypothesis, mutagenesis studies have shown that while some genes have the requirement of a canonical TATA box, others use promoters that deviate from the consensus.

Once the TBP and TFB transcription factors are bound to the promoter, transcription is started by recruitment of RNA polymerase. However, relatively little is known about the processes of transcript elongation and termination in Archaea. Archaeal RNA polymerase is known to transcribe through DNA-binding proteins without the aid of elongation factors, similar to the eucaryal Pol III enzyme, and lacks most Pol II elongation factors, with the exception of TFS. Similarly with termination, there are no known archaeal homologues of eucaryal Pol II termination factors. Instead, archaeal transcription termination suggests sequence-directed intrinsic termination as well as factor-mediated termination.

Translation

The translation system of Archaea has hybrid eucaryal and bacterial character, but all of its ribosomal proteins have eucaryal homologues. The ribosomal protein genes of Archaea are organized into multigene clusters that resemble operons of Bacteria, and they generally contain fewer copies of the three rRNA genes – 16S, 23S, and 5S – likely reflecting their relatively slow growth rate compared to well-characterized Bacteria. Some Archaea (e.g., *Haloarcula marismortui*) contain multiple rRNA operons that differ by 3% or more in sequence, consistent with recent acquisition or divergent functions (e.g., activity at different temperatures). Archaea contain a full complement of tRNA (transfer RNA), a fraction of which may contain introns, and aminoacyl–tRNA synthetase genes in their genomes for use of the standard genetic code; however, incorporation of glutamine and asparagine proceeds by modification of the amino acids on the charged tRNAs using GatABC amidotransferase.

Two unusual amino acids present in methanogenic Archaea are selenocysteine and pyrrolysine, although neither appears to be uniquely archaeal. Selenocysteine is inserted at certain opal (UGA) stop codons through a context-dependent suppressor tRNA (Sec-tRNA$^{\mathrm{Sec}}$) and directed by a selenocysteine insertion sequence. Studies of *M. jannaschii* and *M. maripaludis* showed that the insertion sequence was located 3′ to the UGA codon, and the decoding event is more similar to Eucarya. Selenocysteine has been found in proteins including those involved in reduction of carbon dioxide to methane and subunits of hydrogenase catalyzing the utilization of hydrogen. Recent studies of *M. barkeri* showed the presence of the amino acid, pyrrolysine, in the methylamine methyltransferase enzyme. The position of pyrrolysine is at an amber (UAG) codon, and it may be inserted cotranslationally using a specialized amber suppression tRNA.

The existence of a coupled transcription/translation mechanism as commonly observed in bacterial species has been observed in at least one archaeal species, *T. kodakarensis*. Archaeal transcripts are not thought to be modified with 5′-end caps, although primary transcripts, like those of the haloarchaeal bacterio-opsin gene, may be capped *in vitro* using the viral capping enzyme. Some transcripts have also been shown to contain polyadenine tails at their 3′ ends, as found in eucaryal transcripts.

Further, a wide range of posttranslational modifications have been found in archaeal proteins. These include acetylation, amino acid modification, such as hypusination and thiolation, disulfide bond formation, glycosylation, including both N- and O-linked modification, lipid modification, methylation, phosphorylation, and proteolytic processing. Many of these modifications are shared among the three domains of life; however, some, such as the methylation of methyl-coenzyme M reductase and the unique lipid moieties of haloarchaeal proteins, are distinctly archaeal. For protein secretion, the archaeal general secretory (Sec) machinery is a hybrid of eucaryal and bacterial systems. In addition, the twin-arginine (Tat) protein export is also extensively used in some Archaea, especially among haloarchaea, and in contrast to Bacteria, where it is mainly used for secretion of redox proteins.

Archaeal Genomics

Dozens of archaeal genome sequences have been completed and are available in public databases (**Table 3**). Nearly three-quarters of the genomes are from Euryarchaeota, with one-quarter being from Crenarchaeota, and a few from thus far unclassified organisms. Sizes of the sequenced archaeal genomes ranges from less than 0.5 Mbp for the smallest, *Nanoarchaeum equitans*, to over 5.75 Mbp for

Table 3 Table of Archaea with completely sequenced genomes

Organism	*Size*	*GC*	*Shape*	*Motility*	*Oxygen req.*	*Temp. range*	*Salinity req.*
Aeropyrum pernix K1	1.7	56.3	Coccus	Yes	Aerobic	Hyperthermophilic	
Archaeoglobus fulgidus DSM 4304	2.2	48.6	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	
Cenarchaeum symbiosum A	2.0	57.7	Rod	No	Facultative	Psychrophilic	
Caldivirga maquilingensis IC-167	2.1	43.1	Rod	No	Microaerophilic	Hyperthermophilic	
Ferroplasma acidarmanus fer1	1.9	36.5	Rod	No	Anaerobic	Mesophilic	
Haloarcula marismortui ATCC 43049	4.3	61.1	Pleomorphic, disks	Yes	Aerobic	Mesophilic	Extreme
Halobacterium sp. NRC-1	2.6	65.9	Rod	Yes	Facultative	Mesophilic	Extreme
Haloferax volcanii DS2	4.0	65.5	Pleomorphic, disks	Yes	Aerobic	Mesophilic	Moderate
Haloquadratum walsbyi DSM 16790	3.2	47.9	Square	Yes	Facultative	Mesophilic	Extreme
Hyperthermus butylicus DSM 5456	1.7	53.7	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	Moderate
Ignicoccus hospitalis KIN4/I	1.3	56.5	Coccus	Yes	Anaerobic	Hyperthermophilic	
Korarchaeum cryptofilum	1.6	49.0	Ultrathin filament	No	Anaerobic	Thermophile	
Metallosphaera sedula DSM 5348	2.2	46.1	Coccus	No	Aerobic	Thermophilic	
Methanobrevibacter smithii ATCC 35061	1.9	31.0	Rod	No	Anaerobic	Mesophilic	
Methanocaldococcus jannaschii DSM 2661	1.7	31.3	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	Moderate
Methanococcoides burtonii DSM 6242	2.6	40.8	Irregular coccus	Yes	Anaerobic	Psychrophilic	Moderate
Methanococcus aeolicus Nankai-3	1.6	30.0	Irregular coccus	Yes	Anaerobic	Mesophilic	
Methanococcus maripaludis C5	1.8	33.0	Irregular coccus	Yes	Anaerobic	Mesophilic	
M. maripaludis C6	1.7	33.2	Irregular coccus	Yes	Anaerobic	Mesophilic	
M. maripaludis C7	1.8	33.3	Irregular coccus	Yes	Anaerobic	Mesophilic	
M. maripaludis S2	1.7	33.1	Irregular coccus	Yes	Anaerobic	Mesophilic	
Methanococcus vannielii SB	1.7	31.3	Irregular coccus	Yes	Anaerobic	Mesophilic	
Methanocorpusculum labreanum Z	1.8	50.0	Coccus	No	Anaerobic	Mesophilic	
Methanoculleus marisnigri JR1	2.5	62.1	Coccus	Yes	Anaerobic	Mesophilic	
Methanopyrus kandleri AV19	1.7	62.1	Rod	Yes	Anaerobic	Hyperthermophilic	Moderate

(*Continued*)

Table 3 (Continued)

Organism	*Size*	*GC*	*Shape*	*Motility*	*Oxygen req.*	*Temp. range*	*Salinity req.*
Methanoregula boonei 6A8	2.5	54.5	Rod	No	Anaerobic	Mesophilic	
Methanosaeta thermophila PT	1.9	53.6	Coccus	No	Anaerobic	Thermophilic	
Methanosarcina acetivorans C2A	5.7	42.7	Irregular coccus	No	Anaerobic	Mesophilic	
Methanosarcina barkeri Fusaro	4.9	39.2	Coccus	No	Anaerobic	Mesophilic	
Methanosarcina mazei Go1	4.1	41.5	Irregular coccus	No	Anaerobic	Mesophilic	
Methanosphaera stadtmanae DSM 3091	1.8	27.6	Sphere	No	Anaerobic	Mesophilic	
Methanospirillum hungatei JF-1	3.5	45.2	Curved	Yes	Anaerobic	Mesophilic	
Methanothermobacter thermautotrophicus Δ H	1.8	49.5	Cylinder, irregular rod	No	Anaerobic	Thermophilic	
Nanoarchaeum equitans Kin4-M	0.5	31.6	Sphere	No	Anaerobic	Hyperthermophilic	
Natronomonas pharaonis DSM 2160	2.7	63.1	Rod	Yes	Aerobic	Mesophile	Moderate
Nitrosopumilus maritimus SCM1	1.6	34.2	Rod	No	Aerobic	Mesophilic	
Picrophilus torridus DSM 9790	1.5	36.0	Coccus	No	Aerobic	Thermophilic	
Pyrobaculum aerophilum IM2	2.2	51.4	Rod	Yes	Facultative	Hyperthermophilic	
Pyrobaculum arsenaticum DSM 13514	2.1	55.1	Rod	Yes	Anaerobic	Hyperthermophilic	
Pyrobaculum calidifontis JCM 11548	2.0	57.2	Rod	Yes	Facultative	Hyperthermophilic	
Pyrobaculum islandicum DSM 4184	1.8	49.6	Rod	Yes	Anaerobic	Thermophilic	
Pyrococcus abyssi GE5	1.8	44.7	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	
Pyrococcus furiosus DSM 3638	1.9	40.8	Coccus	Yes	Anaerobic	Hyperthermophilic	
Pyrococcus horikoshii OT3	1.7	41.9	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	
Staphylothermus marinus F1	1.6	35.7	Coccus	No	Anaerobic	Hyperthermophilic	
Sulfolobus acidocaldarius DSM 639	2.2	36.7	Coccus	No	Aerobic	Thermophilic	
Sulfolobus solfataricus P2	3.0	35.8	Irregular coccus	No	Aerobic	Hyperthermophilic	
Sulfolobus tokodaii 7	2.7	32.8	Coccus	No	Aerobic	Hyperthermophilic	
Thermococcus kodakarensis KOD1	2.1	52.0	Irregular coccus	Yes	Anaerobic	Hyperthermophilic	
Thermofilum pendens Hrk 5	1.8	57.6	Rod	No	Anaerobic	Hyperthermophilic	
Thermoplasma acidophilum DSM 1728	1.6	46.0	Pleomorphic	Yes	Facultative	Thermophilic	
Thermoplasma volcanium GSS1	1.6	39.9	Pleomorphic	Yes	Facultative	Thermophilic	
Noncultured methanogenic archaeon RC-I	3.2	54.6				Mesophilic	

M. acetivorans. Thermophiles represent the largest group of Archaea with completely sequenced genomes, including a number of hydrothermal vent species and high-temperature methanogens, while mesophilic methanogens and halophiles constitute the next largest groups. Relatively few acidophilic, alkaliphilic, and psychrophilic Archaea are represented so far.

Among the first archaeal genomes to be sequenced were *M.* (originally *Methanococcus*) *jannaschii*, an autotrophic hyperthermophilic methanogen from a deep-sea trench; *Methanothermobacter thermoautotrophicus* (originally *Methanobacterium thermoautotrophicum*), an autotrophic thermophilic methanogen from an anaerobic sewage sludge digester; *Archaeoglobus fulgidus*, a sulfur-metabolizing thermophile; *Pyrococcus horikoshii*, a hyperthermophile from a hydrothermal vent; *A. pernix*, an aerobic hyperthermophile; *Thermoplasma acidophilum*, an acidophilic and slightly thermophilic heterotroph from a coal refuse pile; *Halobacterium* sp. NRC-1, an aerobic halophilic archaeon probably isolated from salt used in food preservation; and *S. solfataricus*, which metabolizes sulfur and grows at high temperature and under acidic conditions. These initial genome sequences reinforced the validity of the three-domain view of life and also revealed complexity in gene histories as a result of lateral gene transfers and unequal evolutionary rates.

Methanocaldococcus jannaschii

The first archaeal genome, for *M. jannaschii* DSM2661 (originally *Methanococcus jannaschii*), an autotrophic archaeon, produced a complete 1.66 Mbp circular chromosome sequence and two (58 and 16 kb) extrachromosomal circles by whole-genome random shotgun sequencing and assembly. It was only the fourth microbial genome of any kind to be determined (only *Haemophilus influenzae*, *Mycoplasma genitalium*, and *Synechocystis* sp. were reported earlier). The genome contained 1738 predicted protein-coding sequences, which were identified as likely genes by statistical analysis. At the time of publication, only 38% of its genes could be assigned a cellular role with high confidence. Most of the genes of *M. jannaschii* involved in transcription, translation, and replication were more similar to those found in Eucarya than in Bacteria, confirming the special evolutionary position of Archaea in the evolutionary tree. However, the majority of genes coding for energy production, cell division, and metabolism were more similar to those found in Bacteria. The genome also encoded 14 proteins containing 18 inteins, which are insertions within proteins that are removed autocatalytically with concomitant religation of flanking sequences.

Methanothermobacter thermautotrophicus

The second archaeon to have a complete genome sequence was for *M. thermautotrophicus* ΔH, a thermophilic methanogen (named *M. thermoautotrophicum* at the time of sequencing). The 1.75 Mbp circular chromosome was sequenced using a novel 'multiplex' whole-genome shotgun sequencing approach involving hybridization to yield multiple sequence ladders in a single experiment, one of the few accomplished in this manner. Of the 1855 open reading frames present, about 46% of the predicted proteins could be assigned putative functions based on similarities to previously sequenced genomes, 28% were related to sequences with unknown functions, and 27% were entirely new. About 54% were most similar to other Archaea, but only 19% had significant matches to *M. jannaschii*, indicating that there was a low degree of conservation among orthologous genes in the two sequenced methanogens. As in the case of *M. jannaschii*, most DNA metabolism, transcription, and translation proteins for *M. thermautotrophicus* were also found to be more similar to eucaryal sequences. Four interrupted genes were identified, three tRNA genes with introns, and one protein gene with an intein.

Archaeoglobus fulgidus

The first sulfur-metabolizing organism to have its genome sequence determined, *A. fulgidus* DSM 4304, yielded a 2.18 Mbp circular genome containing 2436 predicted genes. Like the two methanogenic Archaea sequenced before, the information processing systems were found to be clearly eucaryal and several of the biosynthetic pathways for nucleotides, amino acids, and cofactors were similar to orthologues in the *M. jannaschii* genome. However, the genomes displayed the expected differences, with *A. fulgidus* lacking methanogenic pathways, and coding for environmental sensors, regulatory and transport functions required for sulfur metabolism. About a quarter of the genes encoded functionally uncharacterized yet conserved proteins, two-thirds of which were shared with *M. jannaschii.* Archaeal diversity was however supported by the finding of about 25% new proteins encoded in the genome. None of the *A. fulgidus* genes contained inteins, although five tRNA genes with introns were found.

Pyrococcus horikoshii

The complete genome sequence of the first of three closely related hyperthermophilic *Pyrococcus* species, *P. horikoshii* OT3, was found to be a circle of 1.74 Mbp. The genome sequence was assembled using a physical map and verified by long PCR products amplified from the genomic DNA. A total of 2061 putative genes were found, with 20% related to functional genes and 22% to conserved sequences with unknown function. The majority of genes, almost 60%, were found to be new. The average protein was found to be basic, with a pI of about 8, indicating a possible means of adaptation of hyperthermophilic proteins to high temperature. A number of postgenomic studies have addressed the high radiation resistance of *Pyrococcus* species and a genetic system has been reported for the related *P. furiosus* species. Eleven genes contained inteins and two tRNA genes contained introns.

Aeropyrum pernix

The first strictly aerobic hyperthermophile to have a completed genome sequence, *A. pernix* K1, a member of the Crenarchaeota isolated from a coastal solfataric thermal vent, was found to have a 1.67 Mbp circular genome. The structure of this genome was verified by restriction mapping along the entire length of the genome using PCR amplification from genomic DNA. A total of 2694 predicted genes were identified, 25% of which were related to genes with putative function and about 20% related to the sequences with unknown function. The remaining gene products were novel and did not show any significant similarity to known sequences in the databases when reported. As expected for an aerobic organism, all but one of the genes for the tricarboxylic acid cycle were present. The single exception, α-ketoglutarate dehydrogenase, was functionally replaced by a 2-oxoacid:ferredoxin oxidoreductase. Fourteen introns were also discovered in its tRNA genes.

Thermoplasma acidophilum

The genome of an acidophile that is also a slightly thermophilic member of the Euryachaeota, *T. acidophilum* DSM 1728 (growing best at 59 °C and pH 2), was sequenced and yielded a 1.56 Mbp circular chromosome. The lack of a rigid cell wall and the presence of eucarya-type proteases and chaperones have been of interest in this archaeon. Analysis of the 1509 predicted genes showed typical archaeal characteristics as well as a relatively large fraction of bacterial genes (about 10%) likely acquired through lateral gene transfers. A substantial number of genes similar to the phylogenetically distant Crencharchaeote, *S. solfataricus*, which coinhabits the same environments, were identified, also indicating candidates for laterally transferred genes.

Halobacterium sp. NRC-1

The first complete genome sequence of an extreme halophile was that of *Halobacterium* sp. NRC-1 (ATCC 700922), harboring a dynamic 2.57 Mbp genome with a 2.01 Mbp circular chromosome and two related large extrachromosomal DNA circles, pNRC100, 191 kb, and pNRC200, 365 kb. The genome was found to be GC-rich (65.9%) and contained 91 transposable IS elements, representing 12 families. The *Halobacterium* NRC-1 genome coded 2630 predicted proteins, 36% of which were unrelated to any previously reported. Analysis of the genome sequence showed the presence of pathways for uptake and utilization of amino acids, active sodium–proton antiporter and potassium uptake systems, and sophisticated photosensory and signal transduction pathways. Its DNA replication, transcription, and translation systems resembled more complex eucaryal organisms. Phylogenetic studies showed that a substantial fraction (up to 15%) of genes coded for proteins with similarity to a variety of Bacteria, indicating that it contains a high fraction of laterally transferred genes. Methods have been developed for facile cultivation and genetic manipulation of this aerobic mesophile, including construction of gene knockouts and replacements. As a result of its ease of laboratory culturing and study, it has become a popular model among haloarchaea and for classroom teaching.

Sulfolobus solfataricus

The aerobic crenarchaeote *S. solfataricus* P2 was found to contain a 2.99 Mbp genome on a single chromosome and encode 2977 predicted proteins, 40% of which appear to be archaea-specific, and 12 and 2.3% shared exclusively with Bacteria and Eucarya, respectively. The genome appears to be highly plastic, with over 200 diverse transposable IS elements present, as well as evidence of integrase-mediated insertion events. As in other Archaea, the DNA replication, DNA repair and recombination, cell cycle, and transcription systems were found to be similar to Eucarya and multiple replication origins were also present, some of which may represent integrated plasmids. A genetic system has been developed for *Sulfolobus* species and used as model systems, especially for DNA replication and cell cycling studies.

Nanoarchaeum equitans

The genome of the hyperthermophilic obligate symbiont, *N. equitans* Kin4-M, that grows in coculture with a crenarchaeote, *Ignicoccus*, is the smallest sequenced to date, a circle of only 0.49 Mbp. Phylogenetic analysis has indicated that *N. equitans* may be a very early branching archaeal lineage, representing an entirely new archaeal kingdom (Nanoarchaeota). However, some researchers have reported contradictory results, suggesting that it may be a member of the Euryarchaeota. In either case, *N. equitans* has one of the most compact genomes, with 95% of its genome coding and specifying 550 putative proteins, including those for DNA replication and repair, transcription, and translation. However, genes for many core cellular functions, including lipid, amino acid, and nucleotide biosyntheses, are lacking, suggesting that it obtains many of its biomolecules from *Ignicoccus*.

Methanopyrus kandleri

The genome of *M. kandleri* AV19, which was thought to be a deeply branching archaeon close to the root of the tree of life, was sequenced employing a novel method where genomic DNA served as template and 2′-modified oligonucleotides were used for priming. The genome was found to be a GC-rich (61%) 1.69 Mbp circle coding 1692 predicted proteins. Like those of halophiles, *M. kandleri* proteins show an unusually high content of negatively charged amino acids, an adaptation to its relatively high intracellular salinity. Although phylogenetic analysis using 16S rRNA suggested that it belonged to a very deep branch, genome comparisons indicated that *M. kandleri* consistently groups with other archaeal methanogens. In addition, *M. kandleri* was found to share organization of genes involved in methanogenesis with *M. jannaschii* and *M. thermautotrophicus*, indicating that archaeal methanogens may be monophyletic. *M. kandleri* was found to lack many proteins involved in signaling and regulation of gene expression, and appears to have relatively few genes acquired via lateral transfer.

Methanobrevibacter smithii

The genome of the human gut methanogen, *M. smithii* ATCC 35061, has been completely sequenced and yielded a 1.85 Mbp circular genome. Comparative and functional genomics of this strain indicates that it persists in the gut by (1) production of surface glycans resembling those found in the gut mucosa, (2) regulated expression of adhesin-like proteins, (3) consumption of a variety of fermentation products produced by saccharolytic bacteria, and (4) competition for nitrogenous nutrient pools. The presence of *M. smithii* affects digestion of dietary polysaccharides, and may influence both the efficiency of caloric uptake and nutritional health of the host. Similar organisms have also been identified in human dental caries.

Thermococcus kodakaraensis

T. kodakaraensis KOD1 is a sulfur-reducing hyperthermophilic Euryarchaeote that cohabits environments with *Pyrococcus*. Annotation of the 2.09 Mbp *T. kodakaraensis* genome revealed 2306 putative genes, half of which could be annotated. The presence of transposable genetic elements similar to *Pyrococcus* species suggested transfer of genes between the two related genera. However, a substantial number of genes (about 30%) were absent from *Pyrococcus* and unique to *T. kodakaraensis*. A facile gene knockout system has been developed for postgenomic analysis of this hyperthermophile and it has become a popular model for postgenomic studies among thermophiles.

Methanococcus maripaludis

A popular genetic model among methanogens *M. maripaludis* is a mesophilic, hydrogenotrophic methanogen with a 1.66 Mbp circular genome. Of the 1722 predicted protein-coding genes, 44% could be assigned a function, 48% were conserved but had unknown functions, and 7.5% were unique. Complete pathways for methanogenesis were identified, including hydrogenases and eight selenocysteine-containing proteins. No Orc gene homologue could be identified, implying an unusual replication initiation mechanism, and the inteins present in *M. jannaschii* homologues were absent. A facile genetic system has been developed for this organism.

Korarchaeum cryptofilum

K. cryptofilum OPF8 is a member of a large group of deep-branching unclassified Archaea that may represent an entirely new archaeal kingdom (Korarchaeota). However, the *K. cryptofilum* genome appears to be a hybrid of crenarchaeal and euryarchaeal genes and it is unclear if this is the result of horizontal gene transfer or evidence of a common ancestor. Its genome consists of a single 1.59 Mbp chromosome containing 1617 protein-encoding genes and 45 tRNA genes. Based on genome analysis, the organism is expected to be an obligate anaerobe and grow heterotrophically using peptide and amino acid degradation pathways. The genome lacks complete pathways for *de novo* synthesis of several cofactors, which the organism probably scavenges from its environment.

Biotechnological Applications of Archaea

Biotechnology has long been one of the most important driving forces behind studies of archaeal microorganisms. Since Archaea are often found in extreme environments and are evolutionarily distinct from Bacteria and Eucarya, they serve as an excellent source of novel enzymes and biomolecules. Although the term ‘extremozymes’ is used for enzymes from both archaeal and bacterial extremophiles, archaeal extremozymes have played an important role in biotechnology. The development of PCR in the 1980s using DNA polymerase from the thermophilic bacterium *Thermus aquaticus* was followed by enhancement of the process using archaeal enzymes. PCR enzymes are only one of a group of extremozymes from Archaea that have contributed to the development of chemical, medical, and genomic biotechnology.

Extremozymes

The need for more PCR thermostable enzymes, with higher fidelity (proofreading exonuclease activity) and producing larger products (greater processivity), led to the identification of archaeal extremozymes such as Pwo (from *Pyrococcus woesei*), Pfu (from *P. furiosus*), and Vent (from *Thermococcus litoralis*). Other useful enzymes for molecular biology applications include a thermostable DNA ligase from *Thermococcus*, and restriction–modification enzymes identified in methanogens and thermophiles, including *M. thermautotrophicus* and *M. jannaschii*. A group B DNA topoisomerase V from *M. kandleri*, an enzyme that relaxes both negatively and positively supercoiled DNA, is also in use for facilitating strand separation in DNA sequencing.

Industrial and Agriculture Applications

In industrial settings, archaeal lipases are used both in dairy (e.g., cheese production) and in detergent applications. Thermophile and psychrophile enzymes fill a variety of different needs, the latter being useful for laundry washing at lower temperatures, an energy-saving method. Archaeal proteases are also in use for baking, brewing, meat tenderizing, paper manufacturing, and in contact lens cleaning solutions.

Haloarchaea (along with halophilic Bacteria) have long been used in the fermentation processes involved in the production of fermented foods, for example, Thai fish sauce (Nam Pla). Their enzymes have biodegradative capabilities as well as synthetic potential as well. Thermophile enzymes that are useful include amylases and α-glucosidases for use as flavoring agents, baking, and brewing, while trehaloses may be used in food preparation and as preservatives.

Applications of the photopigments of haloarchaea, for example, bacteriorhodopsin in purple membrane, include holography and light- and color-sensitive photoelectric devices. If predictions are correct, it may permit the development of optical storage devices with the ability to store 50 TB of information on a single disk in future.

Fuels and the Environment

With the limited availability of fossil fuels, several Archaea have been identified as candidates for production of alternative fuels. Potentially, archaeal species will be able to produce hydrogen for hydrogen fuel cells using seawater as a basal culture medium. Extremozymes from other organisms have been tested for improving efficiency of oil and gas production by breaking down guar gum around sand grains at high temperatures, thereby increasing flow. Methanogens naturally produce methane and researchers are attempting to harness this ability to produce biogas through biodegradation of agricultural, domestic, and industrial waste, as well as biomass. Several designs that have been used are a rotating biological contactor, anaerobic baffle reactors, as well as an upflow anaerobic sludge blanket reactor in which wastewater flows from the bottom upward through a blanket and methane is extracted at the top. Thermophilic alcohol dehydrogenases may be harnessed to produce ethanol at high temperatures and under acidic conditions.

Medical Applications

Archaea have been used to develop novel biomedical applications such as the use of polyhydroalkanoates that can make up to 60% of *Haloferax mediterranei* biomass, for fabricating medical plastics. Patents have been issued for the use of archaeal liposomes and genetically modified gas vesicles from *Halobacterium* sp. NRC-1 in vaccine development. Thermophilic chaperones are being developed for improving the stability of vaccines. The structure of archaeal ribosomes is being used for understanding the basis of antibiotic action and computer-aided design of a new generation of antibiotics.

Conclusion

Although Archaea are the most recently described group of microorganisms from a modern molecular phylogenetic perspective, their activities were noted long ago. The finding of their extremophilic lifestyles coupled with their useful properties for biotechnology have led to the blossoming of archaeal research since their recognition as a separate evolutionary and taxonomic branch in 1977 by C. R. Woese and coworkers. Since then, many diverse Archaea have been isolated, and extensive studies have clarified their importance to ecological processes. Recently, culture-independent techniques have extended our appreciation of the widespread occurrence of archaeal species worldwide in many common environments. However, no archaeal pathogens have been confirmed to date, in spite of their association with the human gastrointestinal tract. Investigations have also rapidly expanded our knowledge and understanding of their molecular biology, biochemistry, physiology, and genetics, including the similarity of their information transfer systems to those in higher organisms. Finally, the study of Archaea has provided the foundation for conceptualizing early cellular evolution on planet Earth.

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Autotrophic CO_2 Metabolism

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Defining Statement
Autotrophic Modes of Life
Conversion of Inorganic Carbon to Cell Carbon
Mechanisms for CO_2 Assimilation
Assessment of Distribution of the Different Pathways
Regulation
Further Reading

Glossary

autotrophy Self-feeding; autotrophy is the ability of an organism to synthesize all cell carbon constituents exclusively from inorganic carbon. Therefore, if an autotrophic organism was to be grown in the presence of labeled CO_2, every single carbon in the cell would become labeled (except the ones derived from essential growth factors added to the medium).

carboxylation Is the addition of CO_2 or bicarbonate to another carbon-containing molecule, resulting in a carbon–carbon bond.

CO_2 assimilation Describes the biosynthesis of cell carbon constituents (biomass) starting from carbon dioxide or bicarbonate.

CO_2 fixation Describes the conversion of CO_2 (a gas) to an organic compound containing carbon–carbon bonds as well as the assimilation of this CO_2 fixation product.

heterotrophy Organic compounds are utilized as carbon sources.

inorganic carbon species Used in autotrophy are carbon dioxide (CO_2), bicarbonate (HCO_3^-), but also carbon monoxide (CO) or cyanide (CN^-).

primary production Is the formation of organic compounds from inorganic carbon species using light or chemically derived energy.

Abbreviations

CbbR	Calvin–Benson–Bassham cycle regulator
CFI	carboxylating factor for isocitrate dehydrogenase
NMR	nuclear magnetic resonance
PEP	phosphoenolpyruvate
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/ oxygenase

Defining Statement

Heterotrophic organisms (and this includes humans) generally oxidize their carbon sources completely to carbon dioxide (CO_2). Autotrophy is the ability of an organism to synthesize all cell carbon from CO_2 alone. Therefore, autotrophic organisms are essential for life and autotrophy represents quantitatively the most important biosynthetic process in the overall carbon cycle.

Autotrophic Modes of Life

The biosynthesis of organic carbon starting from inorganic carbon species by autotrophic organisms is a prerequisite to sustain life. The complete oxidation of organic carbon compounds to CO_2 by heterotrophic organisms allows the maximum gain of reducing equivalents, which can be transferred to a terminal electron acceptor such as oxygen (or also to nitrate, sulfate, etc. in microorganisms) for energy conservation. CO_2 fixation by autotrophic organisms refills the organic carbon pool. When CO_2 assimilation is viewed as the reversal of carbon oxidation to CO_2 this process then requires: reducing equivalents and input of energy. Because CO_2 is the sole source of carbon for autotrophic organisms, reducing equivalents and energy cannot be obtained by oxidation of an organic carbon substrate (formate and methanol oxidation and reassimilation of carbon dioxide by some aerobes can be viewed as an exception of autotrophy). Instead, the source of reducing power is provided by inorganic compounds such as water, hydrogen, reduced sulfur compounds or ammonium. Likewise, energy is provided by photosynthesis or by reduction of oxidized inorganic compounds such as oxygen, nitrate, or sulfate. Primary production (using light or chemical energy), therefore, occurs in aerobic as well as in anaerobic environments. Members of all three domains of life, Bacteria, Archaea, and Eukarya, are able to thrive autotrophically. It is important to remember, however, that CO_2 assimilation in Eukarya (quantitatively most important in green plants) is of microbial origin, as the chloroplasts from such organisms are thought to have arisen from a cyanobacterial endosymbiont.

Conversion of Inorganic Carbon to Cell Carbon

Overall Equation

The general equation for cell carbon biosynthesis from CO_2 is the same for any given organism, because the average oxidation level of any cell is close to zero (equal to oxidation state of formaldehyde, CH_2O). However, the amount of ATP required is dependent on the mechanism used for CO_2 assimilation (see below). Therefore, autotrophic CO_2 fixation follows the general reaction scheme:

$$CO_2 + 4H + n\mathrm{ATP} \leftrightarrows [CH_2O] + H_2O + n\mathrm{ADP} + nP_i$$

Synthesis of Central Precursor Metabolites

When considering the biosynthesis of all carbon compounds from CO_2, it is sufficient to understand the synthesis of central precursor metabolites. These are intermediates of central carbon metabolism from which building blocks for polymers are made and include acetyl-CoA, oxaloacetate, 2-oxoglutarate, pyruvate, or 3-phosphoglycerate. Five CO_2 assimilation sequences are known presently: the reductive pentose phosphate cycle (Calvin–Bassham–Benson cycle), the reductive acetyl-CoA pathway (Wood–Ljungdahl pathway), the reductive citric acid cycle (Arnon–Buchanan cycle), the 3-hydroxypropionate/malyl-CoA cycle, and the 3-hydroxypropionate/4-hydroxybutyrate cycle. These autotrophic pathways account for the net synthesis of acetyl-CoA (reductive acetyl-CoA pathway, reductive citric acid cycle, and the 3-hydroxypropionate/4-hydroxybutyrate cycle), pyruvate (3-hydroxypropionate/malyl-CoA cycle), or 3-phosphoglycerate (reductive pentose phosphate cycle) from either CO_2 or bicarbonate. The further conversion of acetyl-CoA to C_3- or C_4-compounds is not discussed in detail in this chapter, but in each case the reductive carboxylation of acetyl-CoA to pyruvate, catalyzed by pyruvate synthase (pyruvate:ferredoxin oxidoreductase), has been proposed. The conversion of pyruvate to the C_4-compound oxaloacetate proceeds either by direct carboxylation (pyruvate carboxylase) or via carboxylation of phosphoenolpyruvate (PEP) catalyzed by either PEP carboxylase or PEP carboxykinase. The C_5-compound 2-oxoglutarate is formed from acetyl-CoA and oxaloacetate by enzymes of the oxidative citric acid cycle or via a (complete or incomplete) reductive citric acid cycle.

CO_2 Concentrating Mechanisms

The concentration of dissolved CO_2 at pH7 under an air atmosphere is only about 10 μmol l^{-1}. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the major CO_2 fixation enzyme, has K_m values for CO_2 that range from 10 to 300 μmol l^{-1}. In addition, RuBisCO has a rather slow turnover even at saturating levels of CO_2; this enzyme also catalyses an apparent wasteful competing oxygenation reaction in the presence of oxygen. Carboxysomes provide a microenvironment for RuBisCO with locally elevated CO_2 (and most likely decreased oxygen) concentrations. The function of these protein microcompartments were first suggested for *Halothiobacillus neapolitanus* (formerly: *Thiobacillus neapolitanus*), an aerobic thiosulfate oxidizer and obligate autotroph. Similar cellular inclusions were found in oxygenic phototrophic cyanobacteria. Carboxysomes consist of several different proteins, among them specific shell proteins, carbonic anhydrase (catalyzing the interconversion of bicarbonate and CO_2), and the majority of the cellular RuBisCO enzyme. The reader is referred to 'Intracellular structures of prokaryotes: Inclusions, compartments and assemblages', which covers the structural and functional aspects of the carboxysome in more detail. In addition to carboxysomes, there are various CO_2/bicarbonate transporters involved in the CO_2 concentration mechanism that deliver bicarbonate into the cell. The rather recently recognized protein-facilitated transport of CO_2 and/or bicarbonate across membranes adds a fascinating aspect to autotrophic CO_2 metabolism. Currently, there are at least five such systems known in cyanobacteria. Independent of the CO_2 assimilation mechanism used by a given organism, CO_2 or bicarbonate has to first enter the cell. Furthermore, the different carboxylating enzymes are specific for either CO_2 or bicarbonate. The rapid interconversion of bicarbonate and CO_2 catalyzed by carbonic anhydrases is at issue as long as carbon flux rates are high enough to make the uncatalyzed interconversion of CO_2/HCO_3^- rate-limiting. These aspects of CO_2 metabolism have not been addressed in most autotrophs.

Evolutionary Aspect

The question as to whether autotrophy is a primitive trait has been controversial. That is, did heterotrophic life processes evolve after the establishment of autotrophy or did heterotrophic life forms capable of metabolism of low-molecular compounds present in the 'primeval soup' precede autotrophy? The speculations, hypotheses, and presentations of the various controversial views on this subject will not be part of this overview.

Mechanisms for CO_2 Assimilation

The assimilation of CO_2 cannot mechanistically be simply a reversal of the oxidation of central metabolic intermediates to CO_2, because (nearly) irreversible steps are usually involved in these oxidative routes. Presently five different pathways for CO_2 assimilation are known. The pathways are listed and described in the order of their discovery. For the figures, each pathway is divided into carboxylation (red), reduction (green), and regeneration (blue) steps. The reductive acetyl-CoA pathway is the only linear, noncyclic route and, therefore, does not include a regeneration step.

The Reductive Pentose Phosphate Pathway (Calvin–Bassham–Benson Cycle)

In terms of overall CO_2 assimilated, this pathway for CO_2 fixation is the most important. This is based on the fact that oxygenic phototrophs, such as cyanobacteria, algae, and plants, use the reductive pentose phosphate pathway and such organisms generate the majority of the biomass on earth. The primary carboxylating enzyme, RuBisCO, can comprise up to 50% of the soluble cellular protein in organisms using this cycle and is therefore considered the most abundant protein on earth. The reason for the high abundance of RuBisCO in the cell is its rather slow turnover number of three to five molecules per second (for comparison: carbonic anhydrase, albeit one of the fastest enzymes known, turns over one million molecules per second). In addition, various important mechanistic considerations must be accounted for. During catalysis, an enediol of ribulose-1,5-bisphosphate is formed. This intermediate can not only react with CO_2 but also with oxygen. Carboxylation and subsequent hydrolysis of the C_6 carboxylation intermediate produces two molecules of 3-phosphoglycerate whereas in the case of oxygenation, one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate are formed. Two molecules of the C_2 compound 2-phosphoglycolate generate one molecule of 3-phosphoglycerate but one molecule of CO_2 is lost. This process is called photorespiration and requires additional CO_2 to be fixed by RuBisCO to compensate for the loss of CO_2 during photorespiration. The ability to discriminate between CO_2 and O_2 as substrates is a characteristic feature of individual RuBisCO enzymes. The so-called specificity factor, which describes the ratio of the efficiency of carboxylation and oxygenation of ribulose-1,5-bisphosphate, varies significantly between RuBisCO enzymes from different sources. There has been great effort in understanding how the two substrates, CO_2 and O_2, are discriminated by RuBisCO with the hope to engineer an enzyme which is more efficient toward the carboxylation reaction.

For every three rounds of the reductive pentose phosphate cycle, one molecule of 3-phosphoglycerate is generated from three molecules of CO_2 (**Figure 1**). The cycle starts by carboxylation of three molecules of the C_5 compound ribulose-1,5-bisphosphate catalyzed by RuBisCO. Besides one molecule of 3-phosphoglycerate, which is taken out of the cycle as the primary CO_2

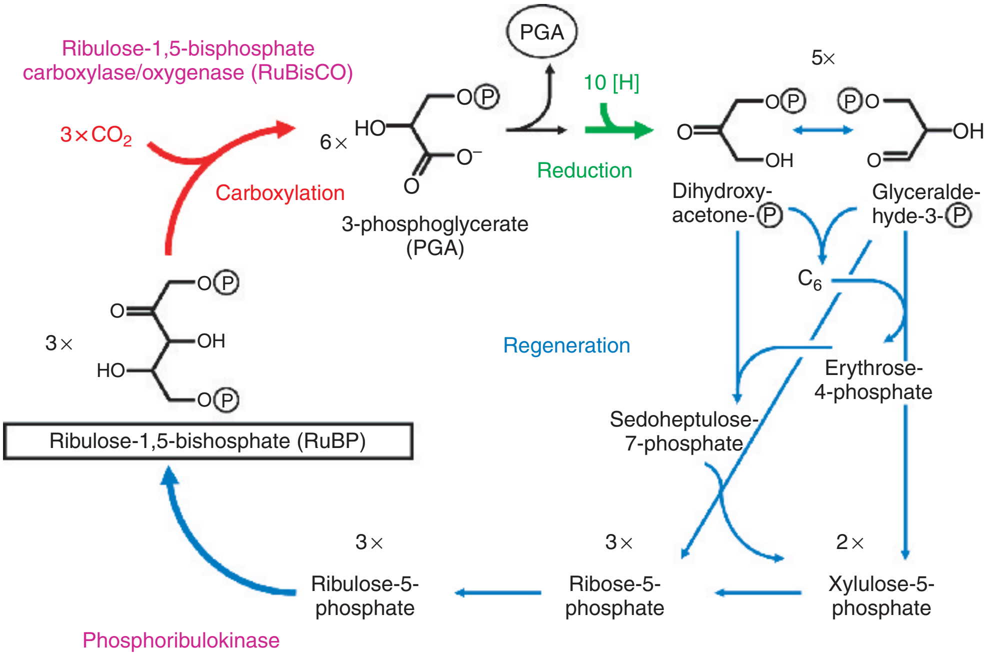

Figure 1 The reductive pentose phosphate cycle (Calvin–Bassham–Benson cycle). The C_6 compound formed from the condensation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate is fructose-1,6-bisphosphate. The primary fixation product of the reductive pentose phosphate cycle is 3-phosphoglycerate (PGA).

fixation product, five more molecules of 3-phosphoglycerate are formed. These five C_3 compounds are used to regenerate three C_5 acceptor molecules in form of ribulose-1,5-bisphosphate.

Regeneration starts by the activation of the second carboxyl group of 3-phosphoglycerate to its phosphate-ester, followed by reduction to the level of the aldehyde catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Triosephosphate isomerase catalyzes the equilibrium between glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The transformation of these five C_3 molecules to two molecules of xylulose-5-phosphate and one molecule of ribose-5-phosphate involves transaldolases and transketolases, which catalyze the transfer of C_3- and C_2- fragments between various activated C_4- (erythrose-4-phosphate), C_5- (xylulose-5-phosphate, ribose-5-phosphate), C_6- (fructose-1,6-bisphosphate), and C_7- (sedoheptulose-7-phosphate) sugar molecules. Ribose-5-phosphate and xylulose-5-phosphate are converted to ribulose-5-phosphate. Phosphoribulokinase, aside from RuBisCO the second unique enzyme of the reductive pentose phosphate cycle, finally activates ribulose-5-phosphate to the CO_2 acceptor molecule ribulose-1,5-bisphosphate and the cycle is closed.

Four forms of RuBisCO enzymes have been recognized. Members of form I, II, III, or IV RuBisCOs have sequence identities of greater than 35% to members of the same class, but less than 30% sequence identity to members of the other classes. Only form I and form II RuBisCO enzymes are involved in autotrophic CO_2 fixation. Form IV RuBisCOs are also referred to as RuBisCO-like proteins, because these enzymes are unable to catalyze carboxylation of ribulose-1,5-bisphosphate and their physiological role is still under investigation. Form III enzymes are bonafide RuBisCOs but occur only in some Archaea; again the physiological role of the form III enzymes is not in autotrophic CO_2 fixation. Form I RuBisCO consists of eight small and eight large subunits and it is the form exclusively present in plants, most algae, and cyanobacteria. In the case of other autotrophic bacteria, which use the reductive pentose phosphate cycle for autotrophic CO_2 fixation, form I, form II, or both forms I and II of RuBisCO are present. Form II RuBisCO consists of only one type of subunit that is similar to the large subunit of form I enzymes.

The Reductive Citric Acid Cycle (Arnon–Buchanan Cycle)

As the name implies, this pathway for autotrophic CO_2 fixation is the reversal of the oxidative pathway (Krebs cycle, tricarboxylic acid cycle) for conversion of acetyl-CoA to two molecules of CO_2. The reductive citric acid cycle has been discovered and initially studied for the green sulfur bacterium *Chlorobium*. More recently, the thermophilic hydrogen-oxidizing bacterium *Hydrogenobacter thermophilus* has become a focus for studying the enzymology of the reductive citric acid cycle. Steps considered essentially irreversible have to be catalyzed by enzymes different from those of the oxidative citric acid cycle. An outline of the pathway is shown in **Figure 2**.

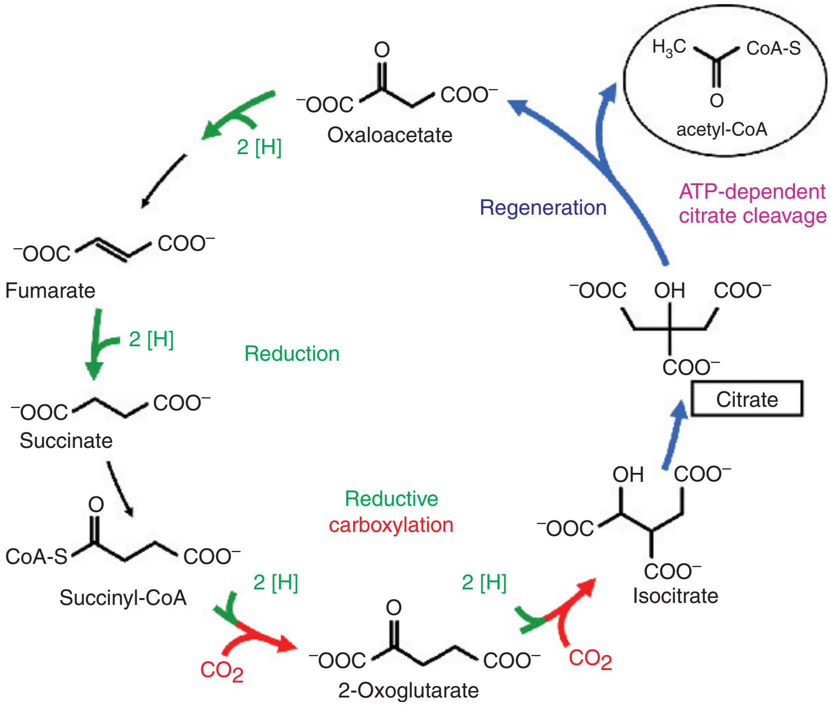

Figure 2 The reductive citric acid cycle (Arnon–Buchanan cycle). The free intermediates of the pathway for members of the *Aquificaceae*, citryl-CoA, and oxalosuccinate, are not shown.

The redox potential of NADH is not sufficient for the reductive carboxylation of succinyl-CoA to 2-oxoglutarate. 2-Oxoglutarate dehydrogenase is, therefore, replaced by 2-oxoglutarate synthase (2-oxoglutarte:ferredoxin oxidoreductase). In addition to using ferredoxin with a more negative redox potential than $NADH/NAD^+$ (which makes the reaction reversible), 2-oxoglutarte synthase is also unrelated to the 2-oxoglutarate dehydrogenase enzyme complex. The same is true for pyruvate synthase (involved in the further assimilation of acetyl-CoA via reductive carboxylation of acetyl-CoA to pyruvate) and pyruvate dehydrogenase complex. 2-Oxoacid dehydrogenases and 2-oxoacid synthases also use different mechanisms. Whereas the 2-oxoacid dehydrogenase complex catalyses one oxidation step with two electrons transferred, 2-oxoacid synthase catalysis involves two electron transfer steps with a radical intermediate. The later enzyme contains iron–sulfur clusters, which can accept one electron at a time and the enzyme is, therefore, oxygen sensitive. However, the enzyme is found and is functional in some aerobic organisms.

The second (nearly) irreversible step in the oxidative citric acid cycle is the condensation of acetyl-CoA and oxaloacetate to form citrate. The reaction catalyzed by citrate synthase is exergonic, because the activation of the carboxyl group from acetyl-CoA is lost and free CoA released. The ATP- and CoA-dependent cleavage of citrate to acetyl-CoA and oxaloacetate during autotrophic CO_2 fixation by the reductive citric acid cycle, consists of two enzymatic activities: first citrate is activated to citryl-CoA (citryl-phosphate as an intermediate is also involved), which – in a second step – is cleaved into acetyl-CoA and oxaloacetate. For the thermophilic hydrogen-oxidizing bacterium *H. thermophilus* these two activities are confined to separate enzymes: citryl-CoA synthetase that consists of two different subunits, catalyzes the first step in the citrate cleavage reaction and is related to succinyl-CoA synthetase (it requires ATP for CoA transfer and releases ADP and inorganic phosphate). A second enzyme, citryl-CoA lyase, cleaves citryl-CoA and forms acetyl-CoA and oxaloacetate. Citryl-CoA lyase is related to citrate synthase. For the green sulfur bacterium *Chlorobium limicola* and *Chlorobium tepidum* both steps are catalyzed by a single enzyme. ATP citrate lyase consists of two different subunits; however, these subunits do not correspond directly to the two separate enzymes of *H. thermophilus*. The large subunit of ATP citrate lyase of *Chlorobium* contains the citrate synthase-related citryl-CoA lyase domain as well as an N-terminal part corresponding to the small subunit of the citryl-CoA synthetase enzyme. The second subunit represents the large subunit of citryl-CoA synthetase. Both subunits of the *C. tepidum* enzyme contribute to the active site of ATP citrate lyase. A fusion protein combining the citrate-activating and citryl-CoA-cleaving domains on a single subunit is found in animals, where ATP citrate lyase plays an important role in fatty acid biosynthesis in the cytosol.

The reversibility of the isocitrate dehydrogenase catalyzing the oxidative decarboxylation of isocitrate to 2-oxoglutarate has been demonstrated for the enzyme from *C. limicola*, even though the equilibrium of the reaction lies on the side of 2-oxoglutartate. In the case of *H. thermophilus* an additional enzyme is required to catalyze the reductive carboxylation of 2-oxoglutarate during CO_2 fixation: 2-oxoglutarate carboxylase is a biotin-containing two-subunit enzyme requiring ATP to form oxalosuccinate (and ADP + P_i) from 2-oxoglutarate and CO_2 (or more likely: bicarbonate). 2-Oxoglutarate carboxylase (formerly named: carboxylating factor for isocitrate dehydrogenase or CFI) is related to pyruvate carboxylase, the enzyme which allows conversion of the C_3-compound pyruvate to the C_4-compound oxaloacetate for the replenishment of the citric acid cycle. A second protein from *H. thermophilus* with almost 50% sequence identity to isocitrate dehydrogenase from *Escherichia coli* has been named oxalosuccinate reductase to indicate its specific role in autotrophic CO_2 fixation. The reductive carboxylation of 2-oxoglutarate to isocitrate by *H. thermophilus* is therefore catalyzed by two enzymes: 2-oxoglutarate carboxylase and oxalosuccinate reductase.

The membrane-bound succinate dehydrogenase complex is replaced by soluble fumarate reductase in the reductive citric acid cycle. Even though, depending on the redox potential of the electron donor, the reduction of fumarate to succinate could be used in energy conservation (fumarate respiration is coupled to the generation of an electrochemical gradient), this has not been observed for autotrophic organisms. An exception may be *Desulfobacter hydrogenophilus*; this sulfate reducer uses the cycle in both directions and fumarate reductase appears to be membrane-bound.

In summary, succinate is formed from the reduction of oxaloacetate and activated to its CoA-ester (**Figure 2**). Two, mechanistically completely different, reductive carboxylation steps follow and isocitrate is formed from succinyl-CoA and two molecules of CO_2. Oxaloacetate is regenerated and acetyl-CoA released as the primary CO_2 fixation product. The last step is brought about by an ATP-dependent citrate cleavage system, which is viewed as the key reaction (sequence) in the reductive citric acid cycle.

The Reductive Acetyl-CoA Pathway (Wood–Ljungdahl Pathway)

The reductive acetyl-CoA pathway is the only linear (noncyclic) pathway for CO_2 fixation. The pathway is linear because acetyl-CoA, the primary CO_2 fixation product, is formed from the direct but independent reduction of two CO_2 molecules: one to the level of a carbonyl group, the other to the level of a methyl group

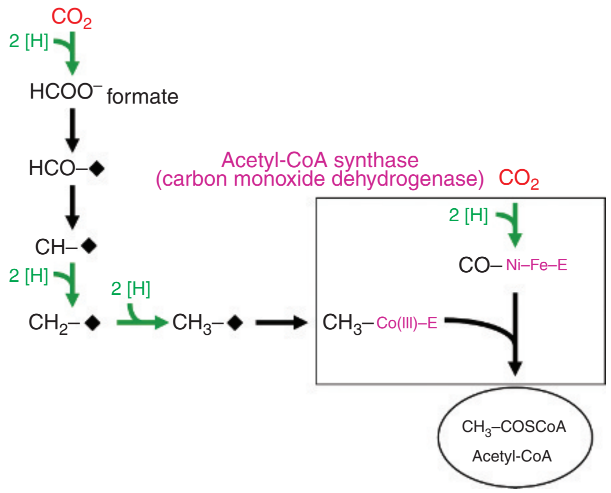

Figure 3 The reductive acetyl-CoA pathway (Wood–Ljungdahl pathway). Because of its metal centers, the key enzyme of the pathway, acetyl-CoA synthase/carbon monoxide dehydrogenase, is extremely oxygen sensitive. ◆ represents a C_1 carrier.

(**Figure 3**). Therefore regeneration of a primary CO_2 acceptor molecule is not required.

One molecule of CO_2 is reduced to an enzyme-bound carbonyl group. This one-step reduction is catalyzed by an enzyme formerly named carbon monoxide dehydrogenase due to this initially observed activity. The same enzyme, however, also impressively catalyzes carbon–carbon bond formation between this enzyme-bound carbonyl group and an enzyme-bound methyl group, and the carbon–sulfur ester bond formation by addition of coenzyme A and was, therefore, renamed acetyl-CoA synthase.

The methyl group is derived from an independent three-step reduction of a second molecule of CO_2. After the first reduction step, catalyzed by formate dehydrogenase, formate is generated as a free intermediate. The subsequent transfer of the formyl group to a C_1 carrier requires the input of energy. Once bound to the C_1 carrier, water is released and the methenyl group reduced in two steps to the methyl group (which corresponds to the oxidation level of methanol). A methyltransferase passes the methyl group from the C_1 carrier on to the corrinoid cofactor of acetyl-CoA synthase, where it will become the methyl group of acetyl-CoA. The nature of the C_1 carriers as well as the electron donors for the reduction steps differ in various organisms using the reductive acetyl-CoA pathway.

Acetogens are eubacteria which form acetate as the main or only product of metabolism. They use the reductive acetyl-CoA pathway for (1) acetyl-CoA synthesis for carbon assimilation from C_1 compounds (including CO_2, carbon monoxide, and formate) as well as for (2) acetate synthesis and energy conservation from a variety of substrates. Autotrophic growth of acetogens is possible, which means that acetyl-CoA synthesis from CO_2 (with hydrogen as electron donor) requires less than 1 ATP, because only 1 ATP is gained by conversion of acetyl-CoA to acetate. The pathway was discovered for acetogens during heterotrophic growth with glucose, where CO_2 is formed and then used as an electron acceptor (see scheme below). All aspects of acetogenesis are covered in detail in 'Acetogenesis'. In the case of acetogens the C_1 carrier is tetrahydrofolate.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H^+ + 8e^-$$

$$2CO_2 + 8H^+ + 8e^- \rightarrow CH_3COOH + 2H_2O$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH$$

Methanogens are archaea which form methane as a metabolic product. Many of them also use CO_2 as a carbon source as well as an electron acceptor. For methanogens, reduction of CO_2 results in the formation of methane instead of acetate as for acetogens. The reductive acetyl-CoA pathway is also required for nonautotrophic methanogenic growth with methylated compounds (methanol, methylamines, etc.) for cell carbon biosynthesis. During methanogenic growth on acetate, acetyl-CoA synthase functions in reverse for acetyl-CoA cleavage, the methyl group is reduced to methane, whereas the carbon group is oxidized to CO_2. For archaeal methanogens, different pterin-based C_1 carriers have been described (e.g., methanopterin, sarcinopterin). In addition, formate is not a free intermediate of the pathway, but instead reduction of CO_2 in the methyl branch of the pathway leads to formyl-methanofuran. The formyl group is subsequently transferred to the pterin-based C_1 carrier and further reduced to the methyl group.

Some sulfate reducers also use the reductive acetyl-CoA pathway for CO_2 assimilation. The same pathway is used in reverse during growth on acetate; acetate is oxidized completely to CO_2 and the electrons are transferred to sulfate (and hydrogen sulfide is formed).

The 3-Hydroxypropionate/Malyl-CoA Cycle

A general outline for the 3-hydroxypropionate cycle is shown in **Figure 4(a)**, indicating the reaction sequences that are shared by two (otherwise distinct) CO_2 assimilation pathways: the 3-hydroxypropionate/malyl-CoA cycle (studied for the green nonsulfur bacterium *Chloroflexus aurantiacus*) and the 3-hydroxypropionate/4-hydroxybutyrate cycle (studied for the thermophilic archaeum *Metallosphaera sedula*). Surprisingly, several enzymes involved in these common steps of both pathways, catalyzing equivalent reactions, are nonhomologous, that is, unrelated. This then suggests an independent origin for the two pathways. Therefore, they are discussed as separate entities.

The primary carboxylating enzymes of the 3-hydroxypropionate/malyl-CoA cycle are acetyl-CoA and

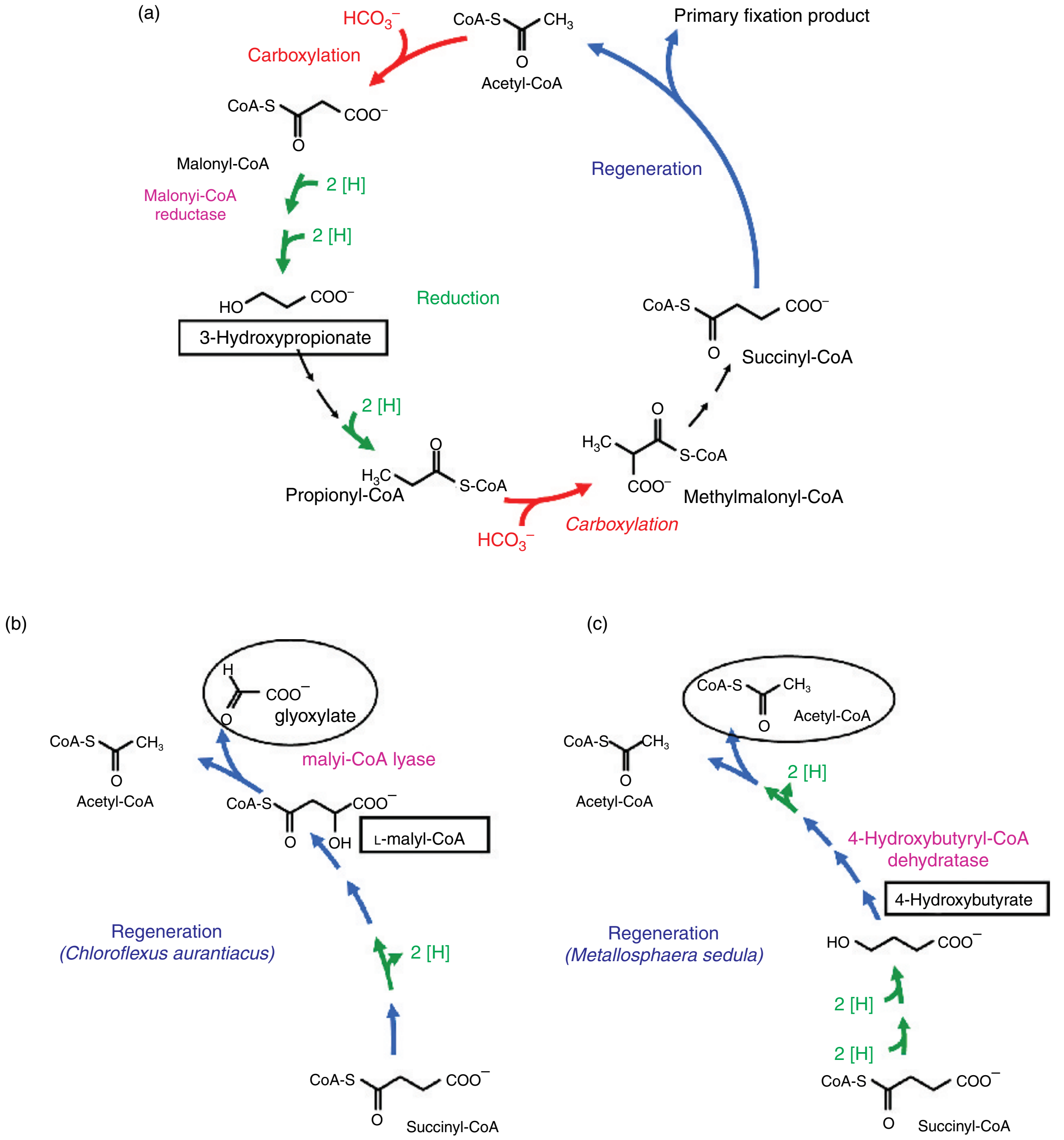
(a)
HCO_3^-
Carboxylation
CoA-S
CH_3
O
Acetyl-CoA
Primary fixation product
CoA-S
COO^-
O
Malonyl-CoA
Malonyi-CoA reductase
2 [H]
2 [H]
Regeneration
HO
COO^-
Reduction
3-Hydroxypropionate
CoA-S
COO^-
O
Succinyl-CoA
2 [H]
O
H_3C
S-CoA
COO^-
Methylmalonyl-CoA
O
H_3C
Propionyl-CoA
S-CoA
Carboxylation
HCO_3^-
(b)
H
COO^-
O
glyoxylate
CoA-S
CH_3
O
Acetyl-CoA
malyi-CoA lyase
CoA-S
COO^-
O
OH
L-malyl-CoA
Regeneration
(Chloroflexus aurantiacus)
2 [H]
CoA-S
COO^-
O
Succinyl-CoA
(c)
CoA-S
CH_3
O
Acetyl-CoA
CoA-S
CH_3
O
Acetyl-CoA
2 [H]
4-Hydroxybutyryl-CoA dehydratase
4-Hydroxybutyrate
Regeneration
(Metallosphaera sedula)
HO
COO^-
2 [H]
2 [H]
CoA-S
COO^-
O
Succinyl-CoA

Figure 4 The 3 hydoxypropionate/malyl-CoA cycle and the 3-hydroxypropionate/4-hydroxybutyrate cycle. (a) General outline of the 3-hydroxypropionate cycle. Note that even though the reactions shown are common for both pathways, most of the enzymes involved in the reductive conversion of malonyl-CoA to propionyl-CoA are unrelated for *Chloroflexus aurantiacus* and *Metallosphaera sedula*. The two-step reduction of malonyl-CoA to 3-hydroxypropionate is catalyzed by one (bifunctional) enzyme and the further reductive conversion to propionyl-CoA involves the trifunctional propionyl-CoA synthase in the case of *C. aurantiacus*. (b) The 3-hydroxypropionate/malyl-CoA lyase cycle as studied in the green phototrophic nonsulfur bacterium *C. aurantiacus*. (c) The 3-hydroxypropionate/4-hydroxybutyrate cycle as studied in the microaerophilic and thermophilic crenarchaeota *M. sedula*.

propionyl-CoA carboxylase. These biotin-dependent enzymes require ATP for catalysis (forming ADP and inorganic phosphate) and use bicarbonate as the inorganic carbon species instead of CO_2.

Carboxylation of acetyl-CoA forms malonyl-CoA. The activated carboxyl group of malonyl-CoA is reduced completely to form the methyl group of propionyl-CoA. This conversion formally requires five enzymatic reactions: reduction of malonyl-CoA to malonate semialdehyde, reduction of malonate semialdehyde to 3-hydroxypropionate, activation of 3-hydroxypropionate to its CoA-ester, dehydration of 3-hydroxypropionyl-CoA to acrylyl-CoA, and finally reduction of acrylyl-CoA to propionyl-CoA. Amazingly, for *C. aurantiacus* these steps are catalyzed by only two proteins. Malonyl-CoA reductase is an unusual protein with only limited sequence identity (and only over a short stretch of the protein) to short-chain alcohol dehydrogenases related to FabG (3-ketoacyl-(acyl-carrier-protein) reductase). Malonyl-CoA reductase catalyzes the two-step reductive conversion of malonyl-CoA to 3-hydroxypropionate using NADPH as the source for reducing equivalents. Propionyl-CoA synthase is a fusion protein containing three functional domains: an acyl-CoA synthetase domain responsible for the activation of 3-hydroxypropionate to its CoA-ester, an enoyl-CoA reductase domain eliminating water from 3-hydroxypropionyl-CoA thereby forming acrylyl-CoA, and an enoyl-CoA reductase domain reducing acrylyl-CoA to propionyl-CoA using NADPH as the source for reducing equivalents.

Carboxylation of propionyl-CoA forms (*S*)-methylmalonyl-CoA. Carbon skeleton rearrangement involves methylmalonyl-CoA epimerase and (*R*)-methylmalonyl-CoA mutase and yields succinyl-CoA. In the 3-hydroxypropionate/malyl-CoA cycle, succinyl-CoA is oxidatively converted to L-malyl-CoA (therefore the name of the pathway) involving enzymes of the citric acid cycle as well as succinyl-CoA:malate CoA transferase (**Figure 4(b)**). L-Malyl-CoA lyase, a key enzyme of this pathway, regenerates acetyl-CoA and yields glyoxylate as the primary CO_2 fixation product. Glyoxylate has to be converted to central biosynthetic intermediate for further assimilation. The assimilation of glyoxylate for *C. aurantiacus* requires a second cycle. Propionyl-CoA is formed by carboxylation of acetyl-CoA and reductive conversion of malonyl-CoA via 3-hydroxypropionate as described (**Figure 4(a)**). Glyoxylate then condenses with propionyl-CoA to form erythro-β-methylmalonyl-CoA, a step which is catalyzed by L-malyl-CoA lyase, the final enzyme of the first cycle. β-Methylmalonyl-CoA is dehydrated to mesaconyl-CoA (2-methylfumaryl-CoA) and a CoA transferase transfers the CoA moiety from one end of mesaconyl-CoA to the other side of the molecule, forming 3-methylfumaryl-CoA (G. Fuchs, personal communication). Hydration of 3-methylfumaryl-CoA yields (*S*)-citramalyl-CoA, which is cleaved into acetyl-CoA and pyruvate. Therefore, in the second cycle, propionyl-CoA and glyoxylate have been converted to acetyl-CoA (from which propionyl-CoA is formed) and the secondary CO_2 fixation product pyruvate, a central intermediate from which cell carbon biosynthesis can proceed by conventional reactions.

The 3-Hydroxypropionate/4-Hydroxypropionate Cycle

As mentioned earlier, this CO_2 assimilation pathway shares intermediates with the 3-hydroxypropionate/malyl-CoA cycle – most notably: 3-hydroxypropionate (**Figure 4(a)**). The enzymes involved in the transformation of these common intermediates, however, are different for the two pathways. In the case of *M. sedula*, using the 3-hydroxypropionate/4-hydroxybutyrate cycle, probably five individual enzymes are required for the reductive conversion of malonyl-CoA to propionyl-CoA (**Figure 4(a)**) compared to only two (fusion) proteins for *C. aurantiacus*. Furthermore, NADPH-dependent malonyl-CoA reductase of *M. sedula* (catalyzing only a one-step reduction of malonyl-CoA to malonate semialdehyde) is unrelated to the enzyme from *C. aurantiacus* (catalyzing a two-step reduction of malonyl-CoA to 3-hydroxypropionate). Instead, malonyl-CoA reductase from *M. sedula* is homologous to aspartate semialdehyde dehydrogenases. Likewise, acrylyl-CoA reductase of *M. sedula* shares very limited sequence identity (centered around a conserved NADPH-binding site) with the enoyl-CoA reductase domain of the trifunctional propionyl-CoA synthase from *C. aurantiacus* catalyzing the same reaction (unpublished results). Carboxylation of propionyl-CoA is catalyzed by a biotin/ATP-dependent and bifunctional acetyl-CoA/propionyl-CoA carboxylase. Succinyl-CoA is formed by carbon skeleton rearrangement of methylmalonyl-CoA.

From here on out the 3-hydroxypropionate/4-hydroxybutyrate cycle differs completely from the 3-hydroxypropionate/malyl-CoA cycle. The reductive conversion of succinyl-CoA to two molecules of acetyl-CoA regenerates the primary CO_2 fixation acceptor molecule, acetyl-CoA; the second acetyl-CoA molecule represents the primary CO_2 fixation product (**Figure 4(c)**). This reaction sequence involves several interesting reactions: succinyl-CoA is reduced with NADPH to succinate semialdehyde, a reaction catalyzed by the same enzyme that reduces malonyl-CoA to malonate semialdehyde earlier in the pathway. Succinate semialdehyde is further reduced to the characteristic intermediate 4-hydroxybutyrate (therefore the name of the pathway) which is activated to its CoA-ester. The 3-hydroxypropionate/4-hydroxybutyrate cycle was discovered after detection of 4-hydroxybutyryl-CoA dehydratase activity in cell extracts of autotrophically grown *M. sedula*. The enzyme catalyzes the challenging elimination of water from an activated 4-hydroxyacid by a ketyl radical mechanism and had been discovered and previously only been described during fermentation by certain clostridia. 4-Hydroxybutyryl-CoA dehydratase yields crotonyl-CoA. The oxidative conversion of crotonyl-CoA to two molecules of acetyl-CoA via 3-hydroxybutyrate and acetoacetyl-CoA involves reactions known from other common metabolic routes.

Other CO_2 Assimilation Pathways

It is very likely that other mechanisms for CO_2 fixation will be described, for example, for extremophiles which will be discovered in the future. So far a considerable number of extremophiles have turn out to be autotrophs using chemical energy (mainly anaerobic respiration) for growth. The description of the 3-hydroxypropionate/4-hydroxybutyrate cycle also shows that parts of one CO_2 fixation pathway may be combined with other reaction sequences to create a new cycle.

There are already some reports in the literature of organisms for which all of the earlier described mechanisms can be ruled out and the question remains how these autotrophs assimilate CO_2. For the obligate autotrophic anaerobic archaeum *Ignicoccus hospitalis* acetyl-CoA was proposed as the initial CO_2 acceptor, but until now it was not clear how acetyl-CoA is (re)generated. However, very recent data indicate that acetyl-CoA is formed by reductive conversion of succinyl-CoA as described for the second part of the 3-hydroxypropionate/4-hydroxybutyrtae cycle involving 4-hydroxybutyryl-CoA hydratase (H. Huber and G. Fuchs, personal communication). Other examples are members of the Pyrodictiaceae for which the mechanism of CO_2 fixation is still unknown.

Assessment of Distribution of the Different Pathways

Key Enzymes

Key enzymes of pathways catalyze reactions yielding or utilizing unique intermediates in metabolism that are characteristic for that particular pathway. Key enzymes can, therefore, be used as markers and their detection provide an excellent indication for the presence of an entire reaction sequence involving other enzymes that are shared with more common pathways of central carbon metabolism.

The key enzymes of the reductive pentose phosphate cycle are phosphoribulokinase and RuBisCO (**Figure 1**). Phosphoribokinase catalyzes the ATP-dependent activation of ribulose-5-phosphate to ribulose-1,5-bisphosphate, which is the primary CO_2 acceptor molecule of the reductive pentose phosphate cycle. RuBisCO, of course, is the carboxylating enzyme that uses ribulose-1,5-bisphospate as its substrate, forming two molecules of 3-phosphoglycerate.

The key reaction sequence of the reductive citric acid cycle is the ATP-dependent conversion of citrate to acetyl-CoA and oxaloacetate (**Figure 2**). This reaction is catalyzed by ATP citrate lyase or two enzymes: citryl-CoA lyase and citryl-CoA synthetase, depending on the organism. Enzymes of both ATP-dependent citrate cleavage systems are related. In addition, pyruvate and 2-oxoglutarate synthase are required for the reductive carboxylation of acetyl-CoA and succinyl-CoA.

The key enzyme of the reductive acetyl-CoA pathway is acetyl-CoA synthase/carbon monoxide dehydrogenase, which catalyzes the reduction of CO_2 to an enzyme-bond CO intermediate (**Figure 3**). The enzyme also catalyzes formation of acetyl-CoA from the enzyme-bound carbonyl group, an enzyme-bound methyl group, and coenzyme A. In addition, pyruvate synthase is required for the functioning of the reductive acetyl-CoA pathway for further assimilation of the primary CO_2 fixation product acetyl-CoA.

There are several reaction sequences which appear to be unique but also common to the 3-hydroxypropionate/malyl-CoA and 3-hydroxypropionate/4-hydroxypropionate cycles (**Figure 4(a)**). Therefore, the enzymes involved in these reactions can all be referred to as key enzymes. The two-step reduction of malonyl-CoA leads to the formation of 3-hydroxypropionate. This characteristic intermediate is further reduced to propionyl-CoA via acrylyl-CoA. Specifically for the 3-hydroxypropionate/malyl-CoA cycle, L-malyl-CoA lyase is the key enzyme, whereas 4-hydroxybutyryl-CoA dehydratase is characteristic for the 3-hydroxypropionate/4-hydroxybutyrate cycle.

Detection of Key Enzymes

The detection of key enzymatic activities in cell extracts of autotrophically grown cells of a particular organism is a prerequisite to assign a particular CO_2 fixation pathway used by a certain species. The specific activity of the key enzyme must be high enough to account for the doubling time of the organism during autotrophic growth. It is likely that the activity is downregulated during growth on an organic substrate (heterotrophic growth) instead of CO_2 (autotrophic growth). Regulation of an activity in facultative autotrophs in response to growth substrate is an excellent indication of the enzyme's proposed role in a particular pathway. In some instances it is also possible to follow an entire reaction sequence using cell extracts. Similarly, short-term labeling studies with $^{14}CO_2$ or other ^{14}C-labeled (suspected) intermediates and cell extracts or cell suspensions have been found to be extremely helpful. As confirmation of a particular CO_2 fixation mechanism used by a given organism, activities of key enzymes of alternate CO_2 assimilation pathways are expected to be absent.

With the advent of complete genome sequences there is the temptation to assign a specific mechanism for CO_2 fixation of an autotrophic organism based on genomic analysis alone. Furthermore, one may even wish to uncover the prevailing CO_2 fixation pathway in a particular habitat, analyzing sequences derived from entire communities (metagenomics). The assignment of the gene encoding for the key enzyme of a pathway is not sufficient. Candidates for all genes involved in the pathway must be assigned. It is not uncommon to find several enzymes of a pathway encoded by genes that are clustered on the genome. To confirm the proposed role of an assigned gene, it is usually cloned, heterologously expressed, and the recombinant enzyme analyzed for its predicted activity. In some cases, enzymes catalyzing subsequent reactions in a pathway are even fused; for example, propionyl-CoA synthase from *C. aurantiacus* of the 3-hydroxypropionate cycle consists of three domains corresponding to enzymes catalyzing the activation of 3-hydroxypropionate to its CoA ester, dehydration of 3-hydroxypropionyl-CoA, and reduction of acrylyl-CoA to propionyl-CoA. For *Roseiflexus* species the gene encoding propionyl-CoA synthase is found clustered with genes encoding malonyl-CoA reductase and the subunits of acetyl-CoA/propionyl-CoA carboxylase; other genes encoding for enzymes required for the 3-hydroxypropionate cycle are found elsewhere on the genome. It is clear that the *Roseiflexus* species use the 3-hydroxypropionate cycle for CO_2 assimilation. This, however, was surprising because isolates of *Roseiflexus* species, which are filamentous anoxygenic phototrophic bacteria related to *C. aurantiacus* (83% sequence identity on the 16S rRNA level), had not been shown to grow autotrophically. It is possible that *Roseiflexus* species might use the 3-hydroxypropionate cycle to fix CO_2, while coassimilating organic carbon compounds at the same time (mixotrophic growth).

More often than not, the assignment of a particular CO_2 assimilation mechanism or the prediction of the capability of a given organism to grow autotrophically, based on genomic data alone, is not straightforward. The following situations are often encountered:

1. Specific genes can be assigned but are not involved in autotrophic growth. A good example is the presence of a gene encoding RuBisCO in many methanogenic archaea. The enzyme represents a form III RuBisCO and is not involved in autotrophic CO_2 assimilation. These organisms use the reductive acetyl-CoA pathway for CO_2 fixation. ATP citrate lyases are present in a variety of organisms including mammals and play a role in fatty acid biosynthesis. Genes for 2-oxoacid synthases (2-oxoacid: ferredoxin oxidoreductase) are found in many genomes, particularly in genomes of strict anaerobic – and at the same time strict heterotrophic – bacteria. The reductive acetyl-CoA pathway and the reductive citric acid cycle can be used exclusively in reverse by some organisms for acetyl-CoA oxidation during heterotrophic growth. 4-Hydroxybutyryl-CoA hydratase, one of the key enzymes of the 3-hydroxypropionate/4-hydroxybutyrate cycle, was first discovered in *Clostridium aminobutyricum* where it is involved in fermentation.
2. Not all (required) genes of a CO_2 assimilation pathway can be assigned. Although ATP citrate lyase activity (albeit low) was detected in cell extracts of *Thermoproteus tenax* and *Magnetococcus* sp. MC-1 and additional evidence was presented for the functioning of the reductive citric acid cycle in these bacteria, a corresponding gene for ATP citrate lyase is absent in the completed genome sequences. It is not clear, whether another CO_2 assimilation pathway is operating in these organisms or whether the enzyme catalyzing the cleavage of citrate is unrelated to ATP citrate lyase or citryl-CoA lyase. An alternate mechanism for citrate conversion to acetyl-CoA and oxaloacetate has been proposed but experimental evidence is missing. In case of the 3-hydroxypropionate/malyl-CoA cycle, the conversion of malonyl-CoA to propionyl-CoA involves different and in some instances completely unrelated enzymes compared to the 3-hydroxypropionate/4-hydroxybutyrate cycle. Catalysis of identical reactions by unrelated enzymes (convergent evolution) is not unexpected, particularly with members of general enzyme classes, such as dehydrogenases, hydratases, and acyl-CoA synthetases.
3. Genes specific for several pathways appear to be present. The genome of *Archaeoglobus fulgidus* harbors genes encoding proteins related to RuBisCO (form III), acetyl-CoA synthase/carbon monoxide dehydrogenase, and 4-hydroxybutyryl-CoA hydratase. The reductive acetyl-CoA pathway is thought to function as the autotrophic CO_2 assimilation pathway.

Confirmation (or refutation) of a proposed pathway for CO_2 assimilation is possible by long-term ^{13}C-labeling studies as discussed in the following text. This, however, requires rather robust growth of an isolated organism. In case of bacteria and archaea not amenable for mass cultivation, additional evidence might be obtained by studying ^{13}C isotope contents in habitats or microbial consortia which might be indicative of the presence of a specific CO_2 assimilation pathway.

Qualitative Assessment (^{13}C Isotopic Depletion)

Differences in stable ^{13}C isotope content (relative to ^{12}C) between inorganic carbon and organic matter synthesized from CO_2 by autotrophs may be used to suggest a specific mechanism for CO_2 fixation. For example, the depletion of ^{13}C in organic matter relative to CO_2 from which the carbon was derived via the reductive pentose phosphate pathway is due to the preference of RuBisCO for $^{12}CO_2$ relative to $^{13}CO_2$. An isotopic signature of sedimentary organic matter of –20‰ to –30‰ is typical for CO_2 assimilation via the reductive pentose phosphate pathway. In contrast, CO_2 fixation via the reductive citric acid cycle only leads to a depletion of –2‰ to –12‰. For the 3-hydroxypropionate/malyl-CoA cycle a depletion value of –14‰ has been reported. The depletion of ^{13}C carbon of organic matter formed by the reductive acetyl-CoA pathway relative to the $^{13}CO_2$ assimilated is greater than –30‰.

The analysis of stable carbon isotopes can provide insights into carbon fixation mechanisms used (and the type of organisms involved) in a given habitat. This concept has been applied to microbial mats present in the effluent of sulfur-containing hot springs. The ^{13}C isotope content of organic matter of mats close to the source pool was diagnostic for the 3-hydroxypropionate cycle, consistent with the fact that those mats were constructed mainly by *Chloroflexus* species. Mats further downstream formed by both cyanobacteria and *Chloroflexus* had a more typical reductive pentose phosphate cycle signature. However, compound-specific isotope analysis of unique lipids of *Chloroflexus* pointed mainly to CO_2 assimilation (via the 3-hydroxypropionate cycle) in addition to some cross-feeding of photosynthetic products from cyanobacteria by *Chloroflexus*.

Qualitative Assessment (Long-Term *In Vivo* ^{13}C-Tracer Labeling Studies)

A very elegant method has been introduced for analyzing the specific fate of fixed CO_2 into specific positions of central precursor metabolites, reflecting the mechanism of CO_2 assimilation in a given autotrophic organism. Limited amounts of differentially ^{13}C-labeled intermediates of the proposed CO_2 fixation pathway are added as tracers to cultures growing autotrophically. After several generation times, the cells are harvested and major cell constituents are isolated. The ^{13}C-labeling patterns of several building blocks (such as sugars, amino acids, and nucleotides) are determined by quantitative nuclear magnetic resonance (NMR) spectroscopy. Based on known biosynthetic pathways, ^{13}C-labeling patterns of central metabolic metabolites are retraced. It is important to note that the labeling pattern of a particular central metabolite is determined multiple times, because all the different building blocks are derived from one or several of these central metabolites. Therefore, variations in specific biosynthetic pathways in a particular organism can be accounted for. These results are compared with predicted labeling patterns based on the proposed CO_2 assimilation pathway which either leads to the confirmation or falsification of the suggested pathway.

Distribution and Physiological Restrains

There is no clear distribution of the different autotrophic CO_2 fixation pathways according to phylogenetic groups (**Table 1**). However, Archaea as well as strict anaerobic bacteria appear to use a mechanism for CO_2 fixation distinct from the reductive pentose phosphate cycle. The 3-hydroxypropionate cycle does not seem to be used by strict anaerobes.

The diversity of different pathways for the assimilation of inorganic carbon is at first unexpected. However, an alternate way to fix CO_2, other than the very energy-demanding reductive pentose phosphate pathway, was expected for organisms that are only able to gain rather limited energy through their metabolism (e.g., strict anaerobic microorganisms). At the same time, an anaerobic version for CO_2 fixation would not be feasible for aerobic organisms, because some of the enzyme involved in the reductive citric acid cycle and particularly the reductive acetyl-CoA pathway are oxygen-sensitive. (The reductive citric acid cycle, however, is also used by some microaerophilic and even aerobic bacteria with high O_2 respirations rates). In addition to theses two 'anaerobic' versions (reductive acetyl-CoA pathway/reductive citric acid cycle), two versions of high-energy-demanding CO_2 assimilation pathways are now known: the reductive pentose phosphate cycle and the 3-hydroxypropionate cycle. The 3-hydroxypropionate/4-hydroxybutyrate cycle uses 4-hydroxybutyryl-CoA dehydratase, an enzyme containing oxygen labile iron–sulfur clusters and so far the pathway

Table 1 Distribution of the different pathways for CO_2 fixation among various phylogenetic and physiological relevant groups

CO_2 assimilation pathway	*Phylogenetic groups*	*Physiological groups*	*Examples*
Reductive pentose phosphate pathway (Calvin–Bassham–Benson cycle)	Chloroplasts cyanobacteria purple nonsulfur bacteria, purple sulfur bacteria, α-/γ-proteobacteria, etc.	Oxygenic phototrophs, anoxygenic phototrophs, hydrogen-/sulfur-/ammonium-oxidizers nitrate reducers	Plants, algae, *Synechococus* sp., *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Ralstonia eutropha*, *Xanthobacter autorophicus*, *Thiomicrospira crunogena*
Reductive citric acid cycle (Arnon–Buchanan cycle)	Green sulfur bacteria, δ-/ε-proteobacteria, Desulfobacteriaceae, Aquificaceae, etc.	Anoxygenic phototrophs, hydrogen-/sulfur oxidizers, sulfur reducers, sulfate reducers	*Chlorobium tepidum*, *Hydrogenobacter thermophilus*, *Desulfobacter hydrogenophilus*, *Suflurimonas denitrificans*
Reductive acetyl-CoA pathway (Wood–Ljungdahl pathway)	Desulfobacteriaceae, Methanobacteria, etc.	Homoacetogens, methanogens, sulfate reducers	*Moorella thermoacetica*, *Methanothermobacter thermoautotrophicus*, *Desulfobacterium autotrophicum*, *Ferroglobus placidus*
3-Hydroxy-propionate cycle	Green nonsulfur, bacteria, Crenarchaeota (Sulfobaceae), etc.	Anoxygenic phototrophs, thermophilic hydrogen-/sulfur-oxidizers	*Chloroflexus aurantiacus, Metallosphaera sedula*
Unknown			*Ignicoccus*, *Pyrodictium*

has been described for microaerophiles and may also occur in strict anaerobes. Clearly, the presence of oxygen demands adaptation of the CO_2 mechanism used; this is mainly due to the fact that the use of reducing equivalents with low redox potentials makes assimilation of CO_2 more energy efficient, reversible, but at the same time prevents their use in the presence of high oxygen tensions.

So why do even related organisms, for example, different species of sulfur-reducers of the Desulfobacterales, use one 'anaerobic' version for CO_2 fixation over the other (**Table 1**)? Why is the 3-hydroxypropionate cycle generally used by anoxygenic phototrophic green nonsulfur bacteria and the reductive pentose phosphate cycle by phototrophic purple nonsulfur bacteria capable of anaerobic CO_2 fixation? Clearly, there appear to be additional constraints for a particular microorganism to use one pathway rather than another, which are not understood at this point.

The differences between the five (and there might be more) CO_2 assimilation pathways extend beyond the energy requirements and type of electron carriers used. As a matter of fact, those two variables can even differ for the same CO_2 fixation mechanism; for example, the reductive citric acid cycle requires up to two more ATPs to synthesize one molecule of acetyl-CoA from two molecules of CO_2 in the case of *H. thermophilus* compared to *D. hydrogenophilus*. In addition, different electron carriers for the reductive acetyl-CoA pathway are used by methanogens and acetogens. Additional differences between the various CO_2 assimilation pathways are: (1) the requirement for specific cofactors and metals, (2) the type of inorganic carbon species (CO_2 or bicarbonate) assimilated, and (3) and perhaps most importantly, the type of metabolic intermediates through which the carbon passes.

It has been speculated that the 3-hydroxypropionate cycle allows for the simultaneous assimilation of fermentative products, such as acetate or propionate. Likewise, the reductive acetyl-CoA pathway can be used for (co)assimilation of C_1-compounds (carbon monoxide, formaldehyde, formate, methanol, and methyl groups of methylamines). The latter pathway is also reversible and is used for acetate fermentation by methanogens or even some acetogens, provided that the hydrogen partial pressure is kept low. The assimilation of organic compounds and inorganic carbon by parts of the same pathway then would allow for metabolic flexibility such that some of the same enzymes may be used for autotrophic, heterotrophic, or mixotrophic growth. It would be very exciting then, if there were examples of organisms capable of using different CO_2 fixation pathways depending on overall carbon availability. Even for purple nonsulfur bacteria (which use the reductive pentose phosphate cycle) in which autotrophic and heterotrophic growth appeared to be clearly separated at first, RuBisCO has been shown to act in redox-balancing using CO_2 as an electron sink during photoheterotrophic growth on various organic carbon compounds. The unique integration of a particular CO_2 assimilation pathway into the overall carbon metabolism of an organism is therefore an exciting field for further study.

Quantitative Assessment

In terms of total biomass generated, the reductive pentose phosphate pathway is the most important CO_2 assimilation pathway, because it is used by land plants, algae, and cyanobacteria, which are also responsible for maintaining the oxygen level in the atmosphere. With regard to bacterial CO_2 fixation, the contribution of the individual pathways appears to be much more difficult to assess. However, considering the ubiquitous occurrence of cyanobacteria, again there is no doubt that the reductive pentose phosphate pathway contributes the most. Even more importantly, oxygenic photosynthesis allows the synthesis of extensive biomass from CO_2.

In specific habitats, for example, anaerobic or hypothermal, one particular pathway – other than the reductive pentose phosphate cycle – might become dominant. Recent studies, for example, point to the prevalence of the reductive citric acid cycle as the main CO_2 assimilation pathway at hypothermal vents. This pathway is used by proteobacteria of the ε-group, such as *Sulfurimonas denitrificans* or an epibiont of the marine worm *Alvinella pompejana*, contributing significantly to primary production at such sites. Also, it has been suggested that the sulfide-oxidizing uncultured endosymbiont belonging to the γ-proteobacteria, which supplies the deep-sea tube worm *Riftia pachyptila* with fixed carbon, uses the reductive citric acid cycle for CO_2 assimilation, although RuBisCo is also present in the organism and might be used under conditions in which the energy supply is plentiful.

Regulation

Facultative autotrophic organisms are able to also use organic substrates as their carbon source, if these are available. Organic carbon compounds usually become the preferred carbon source over CO_2, because less energy and no exogenous electrons are required for their assimilation. Therefore, the enzymes needed for autotrophic CO_2 fixation are under tight regulation in these facultative autotrophs. Even during mixotrophic growth, for example, when CO_2 and organic carbon are assimilated simultaneously, flow through the CO_2 fixation pathway must be controlled and this is depended on the availability of energy and reducing equivalents.

It is clear that the type of regulation depends on the organism studied as well as the CO_2 fixation mechanism used. The reductive citric acid cycle and the reductive acetyl-CoA pathways may be used in reverse for the oxidation of acetate by some bacteria (sulfate reducers, acetogenic/methanogenic consortia) and even the same enzymes might be involved. Such a scenario would then make posttranslational regulation most likely under those conditions. However, this has not been established at this time.

Limitations to study regulation of the 3-hydroxypropionate cycle at present revolve around the lack of genetic tools for organisms using this pathway for CO_2 assimilation. The activities of key enzymes of this pathway are upregulated during autotrophic versus heterotrophic growth. Of particular interest is the branch point at the level of malonyl-CoA; this C_3 compound is either used for fatty acid synthesis or reduced to 3-hydroxypropionate for further conversion to other central precursor metabolites. The pathway is considered irreversible, making acrylyl-CoA reduction to propionyl-CoA the committed step of the pathway.

Regulation of the reductive pentose phosphate cycle is, therefore, the only CO_2 assimilation pathway studied in some detail. Here the current state of knowledge on the regulation of CO_2 fixation by purple nonsulfur bacteria is briefly discussed. Purple nonsulfur bacteria represent an excellent group of organisms to study carbon metabolism and the molecular basis for its regulation: their enormous metabolic versatility allows them to grow under a variety of different growth modes (anaerobically in the light, aerobically in the dark, and even fermentatively, that is, anaerobically in the dark) using many organic substrates as their carbon source as well as CO_2. In addition, the complete genome sequences of several purple nonsulfur bacteria (*Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Rhodopseudomonas palustris*) have been determined over the past years and genetic tools are in place.

RuBisCO catalyzes the irreversible carboxylation step in the reductive pentose phosphate cycle and is regulated during autotrophic versus heterotrophic growth. A regulator protein, called CbbR (for Calvin–Benson–Bassham cycle regulator), controls the transcription of genes encoding RuBisCO enzymes in all purple nonsulfur bacteria studied and also other bacteria, including nonphototrophic bacteria. As mentioned before, some purple nonsulfur bacteria produce two forms of RuBisCO (e.g., *Rb. sphaeroides*) and the genes encoding both forms I and II are regulated by CbbR. The genes for either form are found in different gene clusters or operons located on distinct chromosomal loci where they are cotranscribed (and coregulated) with additional genes, particularly those that encode enzymes required for the regeneration of ribulose-1,5-bisphosphate, the substrate of RuBisCO. CbbR is a transcriptional regulator that belongs to a class of DNA-binding proteins (LysR-type regulators) that often require the binding of small molecules effectors or coinducers to be active in controlling transcription. It is likely that CbbR binds a metabolite which is present under conditions where CO_2 fixation is desirable but the nature of various positive and negative effector molecules might be different for different bacteria. For *Rp. palustris*, an additional level of regulatory control is added: a three-protein two-component system that is thought to influence the activity of CbbR, the details of this are only beginning to be understood. Oxygen is also sensed and the signal transmitted through the two component RegAB (also called PrrAB) system in *Rhodobacter*. Furthermore, there is differential regulation of the form I and II enzymes and their cognate operons, suggesting additional signals or regulatory elements. Finally, there is also indication of posttranslational regulation and modulation of activity of RuBisCO by certain metabolites, among them the substrate ribulose-1,5-bisphosphate, which is thought to bind tightly to the active site of form I enzymes. Studies on the overall regulation of RuBisCO have already revealed the need for CO_2 fixation by RuBisCO, not only during autotrophic CO_2 assimilation, but also for balancing the redox state of the cell during photoheterotrophic growth.

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Bacillus Subtilis

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Defining Statement
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Glossary

competence Development of the ability to bind and take up DNA from the medium.

engulfment The process by which the developing prespore is completely surrounded by the mother cell.

integrative plasmid A plasmid that cannot replicate autonomously in the host bacterium. It can be maintained by the host bacterium provided it can integrate into the chromosome by homologous recombination.

mother cell One of the two cells formed by the sporulation division. It is required for spore formation but ultimately lyses. It is sometimes called the sporangium.

peptidoglycan The main structural component of the cell wall. It consists of a backbone chain of alternating *N*-acetyl muramic acid and *N*-acetyl glucosamine residues. The muramic acid residues usually have a short peptide side chain; many of these side chains are cross-linked.

phosphotransferase system The PTS catalyzes the coordinated transport and phosphorylation of sugars and related compounds. Phosphoenolpyruvate is the source of the energy and the phosphate for the transport. There are three protein components – HPr, enzyme I, and enzyme II; only enzyme II is specific to the sugar. The different domains of enzyme II are often separate proteins.

prespore One of the two cells formed by the sporulation division. It develops into the spore. It is sometimes called the forespore.

sigma factor A protein that binds to RNA polymerase core enzyme to form RNA polymerase holoenzyme. The sigma factor determines the specificity of the binding of the holoenzyme to promoter sequences in the DNA as a prelude to the initiation of transcription.

SOS response The response to DNA damage. It is also activated during competence development.

spore Dormant, resistant form of a bacterium.

transduction The transfer of genetic material from donor to recipient bacterium mediated by a bacteriophage (a bacterial virus). The DNA is carried within the bacteriophage, replacing some or all of the bacteriophage DNA.

transformation The transfer of genetic material from donor to recipient bacterium in which DNA from the donor is taken up by a competent recipient.

vegetative cell A bacterial cell that is growing actively.

Abbreviations

ABC	ATP-binding cassette
CcpA	catabolite control protein A
FBP	fructose-1,6-bisphosphate
PTS	phosphotransferase system
SASP	small, acid-soluble proteins
TPP	thiamine pyrophosphate

Defining Statement

Bacillus subtilis is a Gram-positive, rod-shaped bacterium that forms heat-resistant, dormant spores. It is not pathogenic. It produces important commercial products. The sequenced genome contains 4 214 630 base pairs. Its genome is easily manipulated genetically. It serves as a model organism for studies of sporulation and of the behavior of low GC Gram-positive bacteria.

Bacillus subtilis is a Gram-positive, rod-shaped bacterium that forms heat-resistant spores. It is commonly found in the soil. It is nonpathogenic. It received its name in 1872 from Ferdinand Cohn, who also demonstrated its ability to form spores that were heat-resistant. It produces several commercially important products, most notably proteases and amylases. In part because of its commercial importance, and more because of the ease of its genetic manipulation, *B. subtilis* has been intensively studied. It has a single circular genome (chromosome). The sequenced genome contains 4 214 630 base pairs (bp) with a 43.5% GC content; it encodes about 4100 proteins. *B. subtilis* is the best characterized of the low GC Gram-positive bacterial species. As is typical of Gram-positive species, it has a cytoplasmic membrane and a thick cell wall, but no outer membrane. This structure contrasts with Gram-negative species, which have a cytoplasmic membrane, a thin cell wall, and an outer membrane.

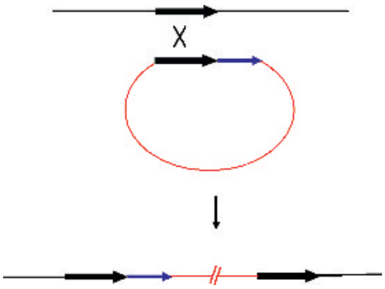

Figure 1 Integration of a plasmid into the chromosome of *Bacillus subtilis* by homologous recombination. Regions of homology on the plasmid and the chromosome are indicated as filled black arrows. The blue arrow indicates a selectable marker, such as an antibiotic resistance determinant, on the plasmid. A single crossover between the regions of homology results in integration of the entire plasmid so that the homologous region is duplicated.

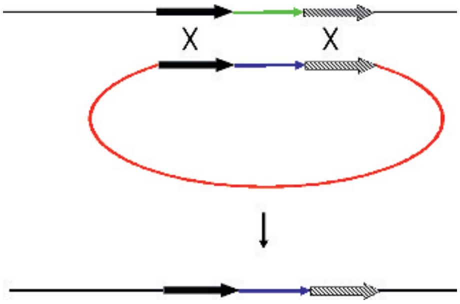

Figure 2 Integration of foreign DNA from a plasmid into the chromosome of *Bacillus subtilis* as the result of double crossover recombination between homologous regions (black and stippled arrows) that flank the foreign DNA (blue arrow). Plasmid (red) and recipient DNA (green) are not present in the recombinant. The blue arrow indicates a selectable marker, such as an antibiotic resistance determinant, on the plasmid.

Genetic Analysis

Genetic analysis of *B. subtilis* was jump-started almost 50 years ago through the discovery of DNA-mediated transformation by Spizizen. In appropriate conditions, *B. subtilis* becomes competent to take DNA from its surroundings (so-called natural competence). If the incoming (donor) DNA shares extensive homology with the recipient chromosome (e.g., if it is derived from another strain of *B. subtilis*), then it can recombine with that chromosome. The net result is genetic exchange between the donor and the recipient strains. Typically, perhaps 1–2% of the donor chromosome ends up in the resultant recombinant strain.

Not all the donor DNA need share homology with the recipient chromosome. Typically, efficient transformation occurs provided the donor DNA has one or two regions of homology of about 500 bp or more. This property makes it possible to introduce foreign DNA into the *B. subtilis* chromosome, for example, genes for antibiotic resistance determinants. It forms the basis of the use of so-called integrative plasmids, which are very widely employed for genetic analysis of *B. subtilis*. Such plasmids replicate in *Escherichia coli* (or some other species), but not in *B. subtilis*. When used to transform *B. subtilis*, they integrate into the chromosome provided they contain DNA that is homologous with the chromosome. When there is a single region of homology between the plasmid and the chromosome, the entire circular plasmid integrates into the chromosome by a single crossover (**Figure 1**). If there are two regions of homology between plasmid and chromosome, it is also possible for a portion of the plasmid to integrate into the chromosome as the result of a double crossover (**Figure 2**).

B. subtilis is also transformable with autonomously replicating plasmids. This can be achieved by natural competence or by transforming protoplasts. The kinetics of transformation by natural competence are unusual, as plasmid trimers are needed to obtain transformants unless the plasmid contains DNA that is homologous to the chromosome; typically, trimers are present as a very minor and variable portion of plasmid preparations.

Generalized transduction was also often used in early studies of *B. subtilis*. The most widely used bacteriophage, PBS1, can carry about 7% of the chromosome from donor to recipient, which is substantially more than the number transferred by transformation. However, the availability of the complete genome sequence has lessened the need for the transfer of large fragments. Most experimenters favor transformation over transduction because of the idiosyncrasies of the transducing phage.

Transposon mutagenesis has proved to be a very effective tool in studies of *B. subtilis*. The most commonly used transposons are derivatives of Tn*10* because of their wide range of sites of insertion, and of Tn*917*. Effective techniques are available to identify DNA sequences adjacent to the sites of transposon insertion, and hence to identify the disrupted gene. Transposon derivatives have been developed with promoter-less reporter genes for use in searches of promoters that respond to particular signals. Integrative plasmids with promoter-less reporters have provided a very effective alternative to transposons. However, both the methods of promoter search are being superseded by microarray analysis.

Nutrients

B. subtilis is able to grow in a minimal medium containing only essential salts, and carbon, nitrogen, and phosphorus sources. A range of mono-, di-, oligo-, and polysaccharides and sugar-derived alcohols can serve as carbon sources, as can amino acids, peptides, and 2-, 3-, and 4-carbon compounds. Nitrate, ammonium ions, urea, amino acids, peptides, and nucleosides can serve as nitrogen sources. Phosphate is the usual phosphorus source in laboratory media, but *B. subtilis* can also use phosphate esters.

B. subtilis is a typical Gram-positive organism with a single lipid bilayer membrane, which acts as a permeability barrier, surrounded by a thick cell wall. Nutrients are taken up from the medium by a variety of transport systems. There are estimated to be at least 15 phosphotransferase systems (PTS) for uptake of different sugars. They are the dominant uptake systems for sugars such as glucose, sucrose, and fructose. Based on the annotated genome sequence, there are perhaps 67 adenosine triphosphate (ATP)-binding cassette permeases, some known to take up sugars, amino acids, or short peptides, but many with unknown substrates. There are maybe 185 transporters that use chemiosmotic energy to drive transport, many with their substrates unknown, several for pumping out rather than in, but a number likely to pump in amino acids, sugars, and other carbon compounds, as well as a variety of ions.

Once inside the cell, carbohydrates are converted to intermediates that are metabolized by the Embden–Meyerhof–Parnas glycolytic pathway, the pentose–phosphate shunt, and/or the tricarboxylic acid (Krebs) cycle. *B. subtilis* respires under aerobic conditions using oxygen as terminal electron acceptor. The respiratory chains that lead to the reduction of oxygen to water involve at least three membrane-located terminal oxidases: cytochrome *aa*3, which is a menaquinone oxidase; cytochrome *caa*3, which is a cytochrome *c* oxidase; and cytochrome *bd*, which also reacts with menaquinone. The terminal oxidases are linked to carbon metabolic pathways by menaquinone reductases coupled to succinate, NADH, and glycerol-3-P. *B. subtilis* was long thought of as a strict aerobe. However, recent studies have shown that it can also grow anaerobically either by fermentation or by respiration with nitrate (or nitrite) as terminal electron acceptor.

Regulation

In nature, *B. subtilis* may face a multiplicity of choices of carbon, nitrogen, and phosphorus sources. It uses a range of regulatory mechanisms to control expression of the genes involved in both catabolic and anabolic pathways. Repressors and activators of transcription predominate among the regulators listed in the annotated genome sequence. The CodY, GlnR, and TnrA proteins are well-studied examples of the many activators and repressors that act in *B. subtilis*. All have more than one regulatory target, and many more in the case of CodY. GlnR and TnrA are regulated by nitrogen availability, while CodY responds to the availability of GTP and branched-chain amino acids. Among their targets, all three regulate the operon for urea utilization – it is repressed by GlnR and CodY, and activated by TnrA. Multiple regulators acting on a particular gene or operon is a common, though not universal, finding with *B. subtilis*.

Two-component signal transduction systems are major mechanisms by which most bacterial species respond to environmental signals. The two components are a sensor kinase and a response regulator. Typically, the kinase has a sensor domain that senses an environmental signal and a catalytic domain containing an autophosphorylatable histidine residue. The response regulator typically has a domain that is phosphorylated on an aspartyl residue by transfer of the phosphate from its associated kinase and an output domain that is often a transcription regulator. They are well represented in *B. subtilis* with over 30 kinases and response regulators. Targets of regulation include chemotaxis, autolysis, competence, degradative enzyme formation, citrate transport, aerobic and anaerobic respiration, and alkaline phosphatase formation. There are variations in the two-component theme, of which the most notable is the phosphorelay associated with Spo0A activation and spore formation (discussed later).

An important regulatory system controls catabolite repression. It differs radically from that in *E. coli* but appears to be present in many Gram-positive species. It also serves to give a hint at the complexities of transcription regulation in *B. subtilis*. Catabolite repression is a global regulatory system in which the presence of a good carbon source, such as glucose, represses expression of the genes for utilization of poor carbon sources even though those carbon sources are present. In *B. subtilis*, the catabolite control protein A (CcpA) represses expression of a number of genes for utilization of poor carbon sources by binding to sites near their promoters. Efficient binding of CcpA, and hence repression, also require a corepressor, the protein HPr. Hpr only fulfills this role when it is phosphorylated on serine residue 46. The kinase that phosphorylates Ser 46 is activated by fructose-1,6-bisphosphate (FBP) – an intermediate of glycolysis. When the glucose concentration is high, the concentration of FBP is high, and HPr becomes phosphorylated and acts as corepressor. When the FBP concentration is low (as with no glucose), HPr is not phosphorylated and does not function as corepressor; hence, catabolite repression is relieved. HPr is a component of the PTS, and the activity of the PTS, reflecting available carbon sources, provides a second control of the ability of HPr to function as a corepressor for catabolite repression.

To transcribe DNA into RNA, RNA polymerase must first bind to particular DNA sequences called promoters. It recognizes particular promoters because of the sigma factor associated with the RNA polymerase. The predominant sigma factor during exponential growth is σ^A. It has what is generally called a housekeeping function, and all the regulators discussed above by and large regulate the action of RNA polymerase containing σ^A. There are a number of other sigma factors that can be considered transcription regulators. They replace σ^A in the RNA polymerase and direct it toward different promoters. They are associated with different regulatory responses. The most studied are as follows: σ^B, associated with the general stress response; σ^D, associated with motility and cell separation; σ^H, associated with the transition state; and σ^F, σ^E, σ^G, and σ^K, associated with spore formation.

Less-well-known mechanisms also serve important regulatory roles in *B. subtilis*. For example, the gene for levan sucrase, *sacB*, is regulated by an antitermination mechanism, as are several other genes. Transcription termination is determined by the structure of recently synthesized RNA, and there is a potential transcription terminator structure between its promoter and the *sacB* structural gene. SacY binds to this RNA region and prevents formation of the termination structure, thus permitting transcription of the structural gene, *sacB*. In the absence of SacY binding, transcription terminates upstream of *sacB*. The ability of SacY to bind is regulated by its phosphorylation state, which responds to sucrose availability through the PTS.

With the structural genes for many tRNA synthetases, it is the cognate tRNA that determines whether transcription does or does not terminate in the region immediately upstream of the structural gene. If the tRNA is uncharged, it binds to this upstream RNA region, preventing formation of the terminator structure and permitting transcription of the structural gene. However, if the tRNA is charged, it can no longer perform this role, transcription terminates, and the structural gene is not transcribed. This same tRNA-mediated regulatory mechanism also operates on a number of genes for amino acid biosynthesis and amino acid transport.

Metabolite-sensing riboswitches have recently been characterized. In these switches, the metabolite interacts directly (no protein is involved) with mRNA located 5′ to structural genes for the synthesis or transport of the metabolite. The binding changes the structure of this 5′ untranslated region. In different systems, it can cause premature termination of transcription and/or it can sequester the ribosome-binding site and so impair translation. For example, thiamine pyrophosphate (TPP) interacts with the 5′ untranslated region upstream of the genes for thiamine synthesis and phosphorylation, causing termination of transcription 5′ to the structural genes; when TPP is not present, the structural genes are transcribed and translated and TPP is synthesized.

Growth and Division

B. subtilis grows at temperatures ranging from 10 to 55 °C, with fastest growth rates at about 42 °C. It forms spores at temperatures up to about 44 °C, depending on strain and medium, but does not become competent for transformation above 37 °C. Most studies use 37 °C. At that temperature, *B. subtilis* grows with a doubling time of about 30 min in a rich medium.

B. subtilis has a single circular chromosome. Chromosome replication is bidirectional. It starts at a fixed origin (0° on the conventional, circular chromosome map) and terminates within a region located at approximately 172°. Complete replication takes about 50 min at 37 °C so that at fast growth rates, chromosome replication is dichotomous; that is to say, a second round of replication starts before the first round finishes.

Recent studies primarily using fluorescence microscopy and a variety of fluorescent tags have made it possible to visualize the behavior of the chromosome during the cell cycle. The process is more readily studied at slow growth rates, where chromosome replication is monochotomous. The chromosome replication machinery, commonly called the replisome, is located at or near midcell. Soon after they are replicated, the two chromosomal origin regions move apart to regions about one quarter of the cell length from each cell end, where they remain for most of the cell cycle. The terminus region remains near midcell. Separation of two terminus regions is thought to be coordinated with completion of division.

The rod-shaped *B. subtilis* cells grow by elongation in their long axis. Peptidoglycan surrounds the cell, defines its shape, and provides the mechanical strength to resist the osmotic pressure caused by the high ion concentrations within the cell (turgor pressure). It constitutes 50–70% of the cell wall. The thick cell wall contains 10–20 layers of peptidoglycan. This contrasts with Gram-negative cell walls that are only 1–2 layers thick. Peptidoglycan is a polymer of *N*-acetyl glucosamine linked to *N*-acetyl muramic acid; the muramic acid has peptide side chains, which are cross-linked to each other. The subunits of the peptidoglycan are assembled in the cytoplasm. At the final stages of assembly (*N*-acetyl glucosamine)–(*N*-acetyl muramic acid)–pentapeptide units are transported across the membrane by a lipid carrier and added to the existing peptidoglycan polymer. The peptidoglycan of the growing cell is a dynamic structure; it is synthesized, modified, and degraded in such a way that the thick cell wall elongates without losing its mechanical strength. Recent results indicate that actin-like proteins form a helix around the cell periphery, which appears to serve as a cytoskeleton. This cytoskeleton, which is probably associated with the inside of the cell membrane, may provide the framework on which peptidoglycan synthesis occurs.

The cell wall also contains anionic polymers, namely phosphate-containing teichoic acids, and under phosphate-limiting conditions, teichuronic acids that do not contain phosphate. The predominant teichoic acids are polymers of either glycerol-phosphate or ribitol-phosphate (depending on strain). They constitute 30–50% of the dry weight of the wall. The teichoic acids appear to be essential for *B. subtilis* and may be involved in cation chelation and/or helping rigidify the wall structure. However, no role has yet been firmly established. In addition to the wall-associated teichoic acids, there is also lipoteichoic acid. The lipoteichoic acid is anchored in the membrane and extends into the wall. This acid is thought to regulate autolysis of the cell wall, which is critical to growth and cell separation after division; however, its role remains poorly understood.

Cell division generally occurs after cells have doubled in length. Division happens precisely at midcell. The length at division depends on growth rate, and fast-growing cells are longer than slow-growing cells. Prior to division, the tubulin-like FtsZ protein polymerizes as an annulus around the cell at the site of division. A series of proteins then assemble at that site, and there is annular growth inward of the cytoplasmic membrane and the cell wall. This inward growth continues until the annulus closes, resulting in two daughter cells. *B. subtilis* has a tendency to grow in chains of cells, depending on medium and strain, and the two daughter cells may only detach from each other sometime after they are formed.

Two mechanisms are known to contribute to the location of the division septum near midcell: nucleiod occlusion and the Min system. The MinC, MinD, and DivIVA proteins are localized at the cell poles and prevent division near those poles. Nucleoid occlusion, as its name suggests, prevents the division septum from bisecting the nucleoid. It remains unclear how the septum is placed so accurately at midcell.

The Transition State

When exponentially growing *B. subtilis* encounters nutrient limitation, it can undergo a series of responses that may help it survive in the changed environment. These responses include secretion of degradative enzymes, synthesis of antibiotics, development of motility, development of competence, and biofilm formation. This period of postexponential activity is often called the transition state. Additionally, *B. subtilis* may go on to form heat-resistant, dormant spores, a defining characteristic of the genus *Bacillus*. Each of the transition-state responses requires major changes in the pattern of gene expression. Several transcription regulators are actively involved in the changes and are known as transition-state regulators. These include AbrB, CodY, ComK, ComP, DegU, SinR, and Spo0A. Typically, these proteins directly or indirectly regulate the expression of a large number of genes. There is considerable overlap between them in the genes they regulate, thus creating a complex pattern that is only partly understood. For example, Spo0A is the master regulator for the entry into spore formation, but it also controls competence development, biofilm formation, antibiotic production, and protease synthesis (though not the synthesis of another extracellular degradative enzyme, amylase). Spo0A directly regulates the transcription of about 120 genes, of which about 80 are repressed and 40 activated. Some 25 genes regulated by Spo0A encode other transcription regulators (including AbrB, CodY, and SinR), and as a consequence, Spo0A indirectly regulates expression of perhaps 500 genes – more than 10% of the *B. subtilis* genome.

Competence

Competence is the physiological state in which bacteria are able to bind and take up transforming DNA. It develops at high cell density and nutritional limitation. The initial stage of the development of competence is the activation of a two-component signal transduction system, ComA and ComP, by quorum sensing. The accumulation of a peptide present in the medium, which is derived from ComX, acts as an indicator of cell density and serves to activate the sensor kinase ComP. ComP then activates ComA by phosphorylation. Activated ComA directs transcription of an operon that includes the gene *comS*. The ComS protein is critical to the stability of ComK, which is the master regulator of competence development. ComK activates transcription of the genes for the 16 or more proteins required for DNA uptake, as well as about 100 other genes. This description gives a bare-bones framework: ComX → ComP → ComA → ComS → ComK → DNA uptake. This framework is subject to complex regulation by a multiplicity of factors including transcription regulators, AbrB, CodY, DegU, and SinR, acting on the various genes involved, as well as by modulators of ComK proteolysis. The DNA that is taken up is single-stranded, and it then typically undergoes homologous recombination with the resident chromosome to yield the transformants.

A striking feature of the development of competence is that it exhibits bistability in which one part of the population (maximally 10–20%) becomes competent and the other part does not. There is no genetic difference between these two populations. However, the two populations differ in many ways and they can be fractionated by density-gradient centrifugation. The critical determinant of the bistability is ComK. During competence development, much of the population expresses *comS*. However, only 10–20% of the population expresses a high level of ComK. Two factors are thought to help establish this bifurcation. First, ComK activates transcription of its own structural gene, *comK*, and this sets up a positive-feedback loop. Second, as competence is developing, there is fluctuation within the population of factors controlling the level of ComK so that only a portion of the population ever reaches a threshold level of ComK that is needed to establish this positive-feedback loop, which leads to accumulation of a high level of ComK and hence the development of competence. Artificial manipulation of the regulators of *comK* transcription or of ComK stability can alter the relative abundance of the two populations. The evolutionary significance of bistability is attracting considerable interest.

Motility, Chemotaxis, and Biofilm Formation

B. subtilis can swim. In this, as in many other ways (notably pathogenesis), it differs from its distant spore-forming relative *Bacillus anthracis*. *B. subtilis* has 10 or more flagella anchored at various sites around the cell (i.e., peritrichous flagella). The flagella can rotate counterclockwise in which case they act in concert to give smooth swimming, or they can rotate clockwise in which case the bacteria tumble. In chemotaxis, the distribution between these two motions is altered such that there is net movement toward attractants or away from repellants.

Particularly during exponential growth in rich media, *B. subtilis* tends to form chains of cell, which lack flagella and so are immotile. These chains break and the bacteria become motile toward the end of exponential growth. Motile bacteria are somewhat more prevalent during exponential growth in minimal media. Expression of genes for flagella biosynthesis depends on a minor RNA polymerase sigma factor, σ^D. This sigma factor also controls genes for autolysins that help break up the bacterial chains: when it is not active, *B. subtilis* forms long chains of cells, which are immotile; when it is active, *B. subtilis* consists primarily of single cells or doublets, which are motile. These two states can coexist, giving another example of bistability.

In standing liquid cultures, wild strains of *B. subtilis* can form a pellicle or biofilm at the liquid–air interface (this ability has been lost by many laboratory strains). In the biofilm, large numbers of bacteria are held together in an extracellular matrix of polysaccharide and protein. Formation of biofilms is repressed by the catabolite repressor CcpA and by the transition-state regulators AbrB and SinR. Intriguingly, SinR activates genes for motility, suggesting that SinR serves as a switch between motility and biofilm formation. SinR is specifically inhibited by another protein, SinI; it is not clear how these various transcription regulators respond to environmental changes.

Antibiotics and Extracellular Enzymes

Formation of antibiotics and extracellular enzymes begins during the transition state. Accumulation of amylase and proteases and of antibiotics in the medium is one of the most characteristic features of the transition state. These disparate entities are often grouped together for that reason. Further, members of both the groups are either of considerable commercial importance or closely related to substances that are of considerable commercial importance. A range of more than 20 antibiotics is produced by different strains of *B. subtilis*, though usually only two or three by any one strain. They are predominantly peptides or modified peptides. They fall into two broad categories: those synthesized by large multienzyme complexes, and those synthesized by ribosomes and then modified after translation.

The ribosomally synthesized antibiotics are lantibiotics. These contain a lanthionine unit formed by the posttranslational reaction of a serine or threonine residue with a cysteine residue, which yields an interresidue thioether linkage. Typically, the lantibiotics kill Gram-positive bacteria by forming voltage-dependent pores in the cytoplasmic membrane. As an example, subtilin is a 32-residue lantibiotic with five lanthionine thioether bridges. The genes for subtilin formation are activated by a two-component system that responds to a quorum-sensing signal; they are repressed by AbrB and require the transition-state sigma factor σ^H. These three mechanisms ensure that induction of antibiotic production occurs during the transition from exponential growth to stationary phase. Antibiotic production requires an immunity mechanism to protect the producing organism from the antibiotic. In the case of subtilin, it is an ATP-binding cassette exporter and a lipoprotein that impairs pore formation.

An example of a nonribosomally synthesized antibiotic is surfactin. It is a lipoheptapeptide and is the most active biosurfactant known. It has a detergent-like action on biological membranes, and antiviral and antimycoplasma activities. It is synthesized by a complex of multidomain enzymes. The corresponding structural genes, *srfA*, *srfB*, and *srfC*, are three of the largest genes in the *B. subtilis* genome, being 10.7, 10.7, and 3.8 kb, respectively. Although it contains these genes encoding the synthetases, the standard laboratory strain of *B. subtilis* does not produce active surfactin, because it contains a mutation in another gene, *sfp*, whose product is required for activation of the surfactin synthetases by phosphopantetheinylation. Resistance to surfactin is provided for the producing organism by an efflux pump. The induction of transcription of *srfA*, *srfB*, and *srfC* is associated with the development of competence. It is activated by the ComA/ComP two-component system responding to accumulation of the ComX peptide (quorum-sensing). Intriguingly, the gene *comS*, whose expression is required for the continued development of competence, is embedded within *srfB* and is transcribed from the *srfA* promoter; it is out of frame with *srfB*.

The two major proteases secreted by *B. subtilis* are a neutral metalloprotease and an alkaline serine protease, known as subtilisin. Subtilisin is of great commercial importance. Uses range from enzyme detergents to studies of protein structure in research laboratories. There are different versions of subtilisin, and these are produced by different strains of *B. subtilis* and its close relatives *Bacillus licheniformis* and *Bacillus amyloliquefaciens*. Note that the favored industrial strains are not the favored laboratory strain, that is, *B. subtilis* 168. Expression of *aprE*, the structural gene for subtilisin, is repressed by AbrB and SinR. These controls link it firmly to the transition state and start of spore formation.

Amylases produced by *B. subtilis* and the same close relatives are also of major commercial importance, notably in the manufacturing of bread and beer. The *B. subtilis amyE* gene encodes an α-amylase. Like the genes for proteases, it is expressed in the transition state after the end of exponential growth. However, the detailed regulatory mechanism is different. It is subject to catabolite repression, being repressed by CcpA. However, its expression is not tied to the start of spore formation.

Spore Formation

Spore formation is the most dramatic response to nutrient depletion. It can be triggered by depletion of carbon, nitrogen, or phosphorus source. The resulting spores are dormant, displaying no detectable metabolism. They are resistant to a variety of stresses that would kill vegetative bacteria. These include heat, noxious chemicals, ultraviolet irradiation, and desiccation. Spore formation takes about 7 h at 37 °C. It begins after transition-state responses such as competence and motility, and bacteria forming spores are neither competent nor motile. In contrast, spores can be formed within biofilms. Secretion of proteases and antibiotic formation are associated with the start of spore formation.

Stages of Spore Formation

The morphological changes during spore formation are similar for all species of *Bacillus* and *Clostridium* that have been studied. The sequence of changes, as established by electron microscopy, has been divided into a series of stages, which are designated with a Roman numeral. These are illustrated in **Figure 3**. Vegetative (growing) bacteria are designated as stage 0. Formation of an axial filament of chromosome extending across the length of the bacterium is designated as stage I. This filament consists of two copies of the chromosome, or one partly replicated chromosome, with a chromosome origin region located near each cell pole. Then, there is an asymmetrically located division resulting in the formation of two unequally sized cells, the larger being the mother cell and the smaller prespore (also called the forespore). The prespore will develop into the mature spore. The mother cell is necessary for this development, but ultimately lyses. Completion of this division is designated as stage II. Remarkably, the chromosomes are not yet equally partitioned into the two cells. The prespore contains the origin-proximal 30% of one chromosome. The mother cell contains the origin-distal 70% of that chromosome as well as another complete chromosome. Following completion of the division septum, a DNA translocase, SpoIIIE, pumps the rest of the chromosome into the

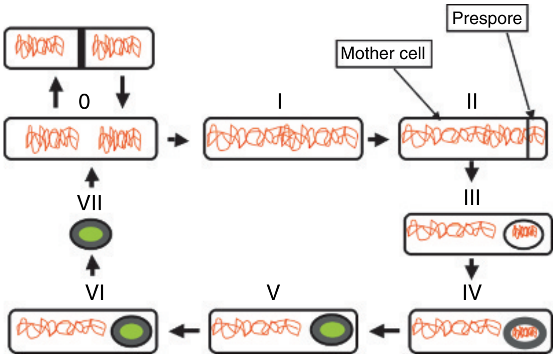

Figure 3 The stages of spore formation. A vegetatively growing cell is defined as stage 0; the top of the diagram indicates the symmetrically located division that occurs during vegetative growth. Stage I is the formation of an axial filament of chromatin that stretches across the long axis of the cell. Completion of the asymmetrically located division is defined as stage II; at this stage, only the origin-proximal 30% of a chromosome is present in the prespore. Completion of engulfment is stage III; by this stage, a complete chromosome is present in the prespore. Stage IV is the synthesis of the primordial germ-cell wall and the cortex between the opposite membranes that surround the prespore (shown in grey). Deposition of layers of coat protein around the prespore is defined as stage V (shown in black). By stage V, the prespore core has contracted to about half its original size and is becoming opaque to stains (indicated by green). Stage VI is completion of maturation, by which time the spore has acquired its full resistance properties. At stage VII, the mother cell lyses and releases the spore into the environment.

prespore. The points of attachment of the septal membrane to the peripheral membrane move toward the cell pole, and the prespore becomes completely engulfed by the mother cell. Completion of engulfment (stage III) results in the prespore being entirely surrounded by the mother cell. Two types of cell wall material, the cortex and the primordial cell wall, are deposited between the opposed membranes that surround the prespore (stage IV). Several layers of different proteins, collectively known as the spore coat, are then assembled on the surface of the prespore (stage V). The developing prespore matures into the resistant spore (stage VI). The prespore core (the prespore cytoplasm) becomes dehydrated during the transition from stage IV to stage VI. The volume of the core is reduced by about half, its density increases, and its optical properties change; the mature spore is refractile by light microscopy and phase-bright by phase-contrast microscopy. The core of the mature spore is impermeable to stains. Finally, the mother cell lyses, releasing the mature spore (stage VII). Because the spore is formed within the mother cell, it is sometimes called an endospore. Spore formation is not an obligatory part of the life cycle of *B. subtilis*, and many viable mutants have been described that cannot form spores. Often they are blocked at a particular stage. SpoII mutants, for example, are blocked at stage II – they form the spore septum but do not proceed to the completion of engulfment; SpoIII mutants complete engulfment but do not proceed further.

The morphological changes during spore formation are intimately linked to a complex pattern of gene expression. The initial changes are funneled through Spo0A, which is the master regulator that controls the start of spore formation (**Figure 4**). They also require the activity of an RNA polymerase sigma factor, σ^{H}, which is specific to the transition state. As soon as the spore septum is formed, two distinct programs of gene expression are set in motion, which are dependent on sporulation-specific sigma factors. One is specific to the prespore and is directed by σ^{F}. The other is specific to the mother cell and is directed by σ^{E}. Following completion of engulfment, two other sigma factors become active – σ^{G} in the prespore and σ^{K} in the mother cell. It is thought that the activities of σ^{F} and σ^{E} are curtailed on completion of engulfment, but it is not clear to what extent this actually happens. The housekeeping sigma, σ^{A}, retains activity in both the prespore and the mother cell, though its activity may be substantially reduced.

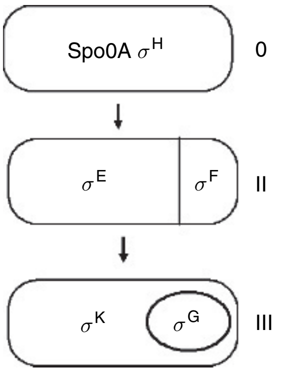

Figure 4 Stages of spore formation at which transcription regulators become active.

The Phosphorelay

Spo0A is activated by phosphorylation via a phosphorelay, which is a more complex version of the classic two-component system. KinA and KinB are the primary kinases for the initiation of sporulation, although three other kinases (KinC, KinD, and KinE) may also play a role. In response to unidentified signals, these kinases phosphorylate the Spo0F protein. The phosphotransferase Spo0B then transfers the phosphate from Spo0F-PO4 to Spo0A (**Figure 5**). The phosphorelay is subject to negative regulation by several phosphatases acting either on Spo0F-PO4 or Spo0A-PO4. Presumably, the complexity of this system enables the organism to integrate a number of internal and external signals before embarking on the energy-demanding path of spore formation. A few of these signals have started to be defined. For example, when cell density is low, the RapA, RapB, and RapE proteins stimulate dephosphorylation of Spo0F-PO4, and hence block Spo0A activation. However, this dephosphorylation is blocked by a quorum-sensing mechanism – when cell density is high, certain peptides, which had been produced by *B. subtilis* and exported into the medium, are imported back into the cell and inhibit Spo0F-PO4 dephosphorylation, thus facilitating activation of

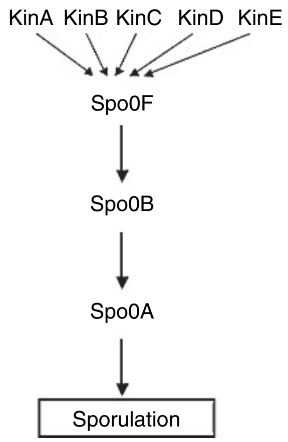

Figure 5 The direction of phosphate flow during activation of Spo0A by the phosphorelay. Arrows indicate the direction of transfer of phosphate residues from histidine kinases via Spo0F and Spo0B so as to activate Spo0A and trigger spore formation.

Spo0A. Other signals go through CodY and Sda. CodY represses the expression of *kinB* as well as the genes encoding the precursor forms of the signaling peptides; a fall in the concentration of GTP and GDP, which is critical to start the sporulation, relieves repression by CodY. Blocks in DNA replication or DNA damage induce expression of the *sda* gene; the Sda protein blocks KinA action and hence Spo0A activation.

Spo0A-PO4 (i.e., activated Spo0A) is necessary for the asymmetrically located sporulation division. It activates transcription of the *spoIIE* locus. This transcription, along with the increased expression of the *ftsZ* locus, which is directed by σ^{H}, results in repositioning of the FtsZ ring from its usual position at midcell to positions near the cell poles. This repositioning occurs via dynamic helical intermediates. The FtsZ rings are formed at both the poles, but normally a septum develops at only one FtsZ ring; the other ring disassembles. The sporulation septum differs from the vegetative division septum by containing much less cell wall material. However, the cause of the difference is not clear. Activated Spo0A is also necessary for the transcription of two other loci that are crucial to subsequent sporulation events – *spoIIA* and *spoIIG*. These loci include the structural genes for the two sigma factors that become active following completion of septation – σ^{F} and σ^{E}.

The *spoIIA* Locus and Activation of σ^{F}

The *spoIIA* locus is a three-gene operon, encoding SpoIIAA, SpoIIAB, and σ^{F}. SpoIIAB is an antisigma factor that binds to σ^{F} and inhibits its action. SpoIIAA is an anti-antisigma factor that binds to SpoIIAB and releases σ^{F} from the inhibitory SpoIIAB–σ^{F} complex. During this process, SpoIIAA becomes phosphorylated by SpoIIAB; SpoIIAA-PO4 is unable to disrupt the SpoIIAB–σ^{F} complex. The other critical player is SpoIIE, which is associated with polar septation and colocalizes with the septum. SpoIIE functions as a phosphatase to dephosphorylate and hence activate SpoIIAA. All these components become available before spore septum formation. However, σ^{F} does not become active until after septum formation. It is thought to be activated as soon as the septum is formed, and it is active only in the prespore. The mechanism of activation has been the subject of intense study, and there is still no complete agreement about it. A critical feature is the volume asymmetry of the division so that the ratio of soluble proteins to membrane-associated proteins is very different in the prespore compared to that in the mother cell or the predivisional organism. This difference is thought to tip the balance so that the membrane-bound SpoIIE wins out in the prespore and σ^{F} becomes active. σ^{F} directs the transcription of about 50 genes, including *spoIIR*, which is required for σ^{E} activation, and *spoIIIG*, which is the structural gene for σ^{G}. The σ^{F}-directed genes fall into two temporal classes – *spoIIR* is an example of the early expressed class and *spoIIIG* of the late. Transcription of *spoIIIG* is subject to complex regulation, which may explain the delay in its expression. It is not clear if other late-expressed genes are subject to the same regulation.

The *spoIIG* Locus and Activation of σ^{E}

The *spoIIG* locus encodes two proteins, SpoIIGA and pro-σ^{E}. Pro-σ^{E} is not active as a sigma factor and needs to be activated by removal of the 27-residue N-terminal pro sequence. It becomes active only in the mother cell. This activation requires SpoIIGA, which is thought to cleave the pro sequence from pro-σ^{E}. Activation also requires the activity of SpoIIR, whose gene is expressed in the prespore from a σ^{F}-directed promoter. Partly overlapping mechanisms contribute to the compartmentalization of σ^{E} activity. One is the directionality of the SpoIIR signal from the prespore. A second is enhanced Spo0A activity in the mother cell following septation, which leads to greatly enhanced *spoIIG* transcription. A third is selective proteolysis in the prespore. Once active, σ^{E} directs the transcription of about 200 genes. (For each of the sporulation-associated sigma factors, estimates by microarray analysis by different laboratories agree on most of the genes that constitute the regulons, but differ somewhat on the absolute number of genes involved; these differences do not affect core conclusions.) This large group of genes (known as the σ^{E} regulon) is subdivided into several groups showing different temporal patterns of expression. Most notably, many genes are repressed by the SpoIIID protein, some are activated by SpoIIID, while many are unaffected; transcription of the *spoIIID* gene is itself directed by σ^{E}. Another regulator also under σ^{E} control, GerR, further subdivides the σ^{E} regulon. This summary helps illustrate the complexity of gene regulation during spore formation.

Genes in the regulon include three, *spoIID*, *spoIIM*, and *spoIIP*, that are required for engulfment and a number of genes encoding coat proteins and proteins required for coat assembly. It includes genes associated with activation of σ^{G} in the prespore and includes the genes for formation of σ^{K}, which is the next sigma factor to become active in the mother cell. The SpoIID, SpoIIM, and SpoIIP proteins are associated with peptidoglycan lytic activity. They are required not only to permit engulfment but also to prevent formation of a second septum at the other end of the organism, opposite to the first septum. It will be remembered that FtsZ forms a ring near both the poles of the cell, but only one of these normally serves as a template for septum formation. However, if SpoIID, SpoIIM, and SpoIIP are not formed, then a second septum is formed at the site of the second FtsZ ring, and development proceeds no further. This behavior is observed, for example, in mutants that lack σ^E. Under these circumstances, the second septum is formed within 10 min of the first. This result would appear to set a severe time constraint on sigma activation. Minimally, within this 10 min, σ^F must become active and direct the transcription of *spoIIR* in the prespore; SpoIIR must trigger the processing of pro-σ^E in the mother cell and σ^E must direct the transcription of *spoIID*, *spoIIM*, and *spoIIP*, whose products must act to prevent the formation of that second septum.

Postengulfment Transcription

Transcription of the structural gene for σ^G, *spoIIIG*, is directed by σ^F in the prespore and depends on σ^E-directed signals from the mother cell as well; it is thought to commence as engulfment is nearing completion. Once activated, σ^G can direct transcription of its own structural gene, setting up a positive-feedback loop to increase its formation. Even a few active molecules could potentially set in motion this self-reinforcing mechanism inappropriately, and there are several controls to prevent premature activation in the mother cell as well as in the prespore. The controls in the prespore are different from those in the mother cell. σ^G is not active when first formed in the prespore. It becomes active only upon completion of engulfment. The controls include σ^E-directed signals from the mother cell, but the mechanism is not understood. The σ^G regulon contains about 100 genes, including one *spoVT* that encodes a regulator of the regulon, which activates some genes and represses some others. The σ^G regulon includes genes for σ^K activation, for the protection of spore DNA (through formation of small, acid-soluble proteins (SASP)), and for germination.

Transcription of the structural gene for σ^K, *sigK*, is directed by σ^E, and so is confined to the mother cell. Once activated, σ^K can direct transcription of its own structural gene, setting up a positive-feedback loop to increase its formation. σ^K is formed as an inactive precursor, pro-σ^K. Processing depends on a σ^G-directed signal from the prespore; it shows no mechanistic similarity to the processing of pro-σ^E. In some strains of *B. subtilis*, including widely used derivatives of the 168 strains, the 5′ and 3′ portions of the *sigK* gene are separated on the chromosome by a 48-kb element known as SKIN. This element is excised by a mechanism that is dependent on σ^E, and so is confined to the mother cell. It is not excised in the developing spore, which is the germ line. In other strains of *B. subtilis* and in other species of *Bacillus*, the *sigK* gene is intact in all cell types so that this mechanism is not essential for spore formation. The σ^K regulon contains about 120 genes. These include *gerE*, which encodes a regulator that represses transcription of some genes in the operon and activates others. The regulon includes genes for coat proteins and for spore maturation.

Spore Resistance

Suspensions of *B. subtilis* spores are completely resistant to 20 min at 80 °C, and about 10% will survive 20 min at 90 °C, whereas vegetative bacteria are completely killed by either treatment. About 10% of dried spores will survive 20 min at 120 °C. Resistance to such wet and dry heat treatments is determined by somewhat different mechanisms. Wet heat resistance is determined largely by the dehydration of the spore core (core is the name used for the cytoplasm of the spore). Cortex formation is essential for the reduction in water content of the core. The accumulation of dipicolinic acid in the core (about 10% of the dry weight) also contributes to dehydration. However, the mechanism of core dehydration is not known. The SASP, which bind to DNA in the spore core, provide some resistance to wet heat; they are critical for resistance to dry heat, where damage to DNA is the lethal event. (However, the mechanism of being killed by wet heat is unclear.)

Resistance to chemicals is provided by different factors, depending on the chemical. The spore coat is the major barrier to many chemicals, including most oxidizing agents. In addition, the membrane surrounding the spore core provides a permeability barrier; the lipids in it are largely immobile, and small molecules pass through it extremely slowly. For a few chemicals, such as formaldehyde and nitrous acid, DNA appears to be the major target where SASP provide protection. Spore resistance to ultraviolet irradiation is also largely determined by the SASP. These proteins change the conformation and photochemistry of the DNA. Efficient DNA repair systems, which become active during germination and outgrowth, are important for protection against all DNA-damaging agents.

Germination and Outgrowth

Spores survive without nutrients and are metabolically dormant. They can survive for hundreds of years, perhaps longer. Yet when they encounter a particular nutrient, they lose their resistance and become metabolically active within minutes; this process is termed germination, and the nutrient (or a few other types of trigger) is the germinant. The particular nutrients that function as germinants vary from species to species. For *B. subtilis*, effective germinants are L-alanine or a mixture of asparagine, glucose, fructose, and K^+ (GFAK). L-Alanine and GFAK have distinct, but structurally similar, receptors in the membrane surrounding the spore core. The germinant reacts with the receptor, and within seconds the spore becomes committed to germinate. The way it happens is not understood mechanistically, but the events in germination are clear: (1) there is a release of protons and cations from the spore; (2) release of dipicolinic acid and its associated cation (predominantly Ca^{2+}); (3) partial rehydration of the spore core; (4) hydrolysis of spore cortex; (5) and swelling of the spore, resulting from further hydration. By this time (a few minutes), the spore has lost its resistance properties and germination is considered complete. It is now that active metabolism resumes, and in a process termed outgrowth, the germinated spore changes into a growing bacterium. Spores contain little or no ATP or NADH, and 3-phosphoglyceric acid serves as energy reserve; degradation of SASP provides a source of amino acids, which also serve as a second energy source. Outgrowth involves a resumption of RNA, protein, and then DNA synthesis, and a change in cell shape; the first vegetative division usually occurs about 2 h after the initiation of germination as long as a growth medium is provided.

Stress Responses

B. subtilis displays a range of responses to different stresses in addition to the transition state/sporulation responses discussed above. The regulatory mechanisms for these responses, which are described below, are highly conserved among low GC Gram-positive bacteria (the Firmicutes). Exposure to a variety of growth-limiting stresses induces the so-called general stress response. This response is mediated by an alternative sigma factor, σ^B, which is regulated by an antisigma factor (RsbW) and an anti-antisigma factor (RsbV), in a manner very similar to that of σ^F regulation during spore formation. Thus, RsbW (like SpoIIAB) also functions as a kinase to phosphorylate RsbV, which is only active as an anti-antisigma factor when it is not phosphorylated (like SpoIIAA). However, the controls of dephosphorylation are different. SpoIIAA-PO4 is dephosphorylated by the phosphatase SpoIIE in response to a morphological signal, the asymmetric sporulation division. There are two phosphatases that act on RsbV-PO4. Both are activated by an environmental signal, but each is subject to its own signals and regulatory system. One responds to environmental stresses such as from acid, ethanol, salt, or heat. The other responds to energy stresses such as from carbon, phosphorus, or oxygen limitation. Deletion of the structural gene for σ^B reduces the ability of *B. subtilis* to survive the various stresses. However, it does not affect spore formation, and the σ^B and σ^F regulatory mechanisms seem largely compartmentalized from each other. The σ^B regulon contains about 200 genes.

Often, there are other responses to particular stresses that accompany the all-encompassing general stress response. For example, a sudden increase in osmotic strength of the medium induces transient expression of the entire general-stress (i.e., σ^B) regulon, and *sigB* mutants are very sensitive to such a shock. Often, the response also involves synthesis of proline as osmoprotectant to almost molar concentrations. This requires radically increased expression of proline biosynthetic genes, directed by σ^A-dependent promoters. High osmotic shock induces expression of regulons under the control of two other sigma factors, σ^M and σ^W; their responses are also induced by the presence of antibiotics in the medium. These observations serve to illustrate the complexity of the responses to a particular environmental shock that can and do occur. They provide a very incomplete picture of that response.

Damage to DNA induces a series of changes known as the SOS response. In *B. subtilis*, the SOS response is also induced during competence development. The response includes increased capacity to repair DNA, mutagenesis, and inhibition of cell division. Genes induced by DNA damage are collectively known as *din* genes (for damage inducible). Many are repressed by the LexA protein (also called DinR). When DNA is damaged, the resulting single-stranded DNA binds to the RecA protein, which then functions as a coprotease to cause LexA to cleave itself and hence inactivate itself. In *B. subtilis*, LexA/RecA appears only partly to control the inhibition of cell division during the SOS response, in contrast to its complete control in *E. coli*.

Different classes of gene have been distinguished as responding to a heat shock in *B. subtilis* based on the regulatory mechanism involved. Class I includes *groEL* and *dnaK*, which are among the most highly conserved heat-shock genes. They are regulated by a repressor, HrcA, that binds to a sequence known as CIRCE located immediately upstream of the open reading frames. HcrA is denatured by heat, which relieves repression. HcrA is then renatured by the action of the GroEL/GroES chaperone, thus restoring repression. Class II genes are regulated by the general stress mechanism, which is described above. Class III genes include *clpC* and *clpE*, encoding ATPases of the Hsp 100 family of heat-shock proteins, and *clpP*, which encodes the proteolytic subunit of the Clp ATP-dependent proteases. The class III genes are regulated by a repressor, CtsR; the regulatory circuit is less understood than that for HcrA, though CtsR is thought also to be denatured by heat. Class IV is the designation given to another group of genes, where the regulatory mechanisms are unknown.

Summary

B. subtilis is the best-studied species of low GC Gram-positive bacteria. It serves as a model for studies of this group, including studies of spore formation in the genera *Bacillus* and *Clostridium*. *B. subtilis* grows in defined, minimal media. It is nonpathogenic. Its genome is sequenced and annotated. There are efficient systems of genetic exchange for *B. subtilis*, making it one of the few species that is readily amenable to genetic manipulation. It displays a wide variety of regulatory mechanisms. Its study continues to yield discoveries that prove applicable to a range of species.

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Bacteriophage (overview)

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Defining Statement
Introduction
Primer on Phage Biology
Importance of Phage
A Closer Look at Certain Aspects of Phage Biology
Overview of Well-Studied Phage Types
Phage Associated with Different Bacterial Hosts
Further Reading

Glossary

adsorption A series of events, culminating in free phage attachment to a bacterium, involving virion movement (via diffusion), virion encounter with a bacterium, virion attachment, and, included by some but not all authors, transfer of the phage genome into the bacterial cytoplasm.

capsid The proteinaceous structure that contains the phage genome.

chronic release Release of mature virions from infected bacteria by extrusion or budding; chronic release neither destroys the bacterium nor ends the phage infection.

free phage A mature phage (virion) particle that is not found associated with a bacterium.

induction (prophage induction) The transition of a lysogenic infection to a productive one, involving expression of numerous prophage genes that otherwise remain quiescent in an uninduced lysogen.

lysis Release of mature virions from an infected bacterium via the destruction of both the bacterium and the phage infection.

lysogeny A phage infection in which phage virions are not produced and the phage genome replicates only so as to continue infecting bacterial progeny as they are produced via binary fission. Typically, this involves phage existence as a prophage that is integrated into the bacterial genome.

lytic cycle A productive infection that ends with bacterial lysis and release of free phage.

lytic phage A phage that displays a lytic cycle given productive infection; an obligately lytic phage cannot display a lysogenic cycle.

productive infection Phage infection that results in production of free phage.

prophage The phage genome during a lysogenic infection (lysogeny).

pseudolysogeny A phage infection that stalls prior to entrance into either lysogenic or productive states, typically due to bacterial starvation. Additional phage phenomena are also described using this terms, however.

temperate phage A phage that is capable of displaying lysogeny, though infections by temperate phage are often productive and do not lead to lysogeny.

virion An infectious viral particle.

Abbreviation

HIV	human immunodeficiency virus

Defining Statement

Bacteriophage (phage) are the viruses of bacteria.

Introduction

Viruses are obligate intracellular parasites and may be categorized in terms of the types of cellular organisms that they infect. Three cell types exist: members of domains Eukarya, Archaea, and Bacteria. The viruses that infect the bacteria, for historical reasons, are called bacteriophage, or phage for short, meaning that they are 'eaters' of bacteria. Since most environments – including our own bodies – contain numerous bacteria, phage are found almost everywhere. Phage biologists have found phage in most environments that can support bacterial growth, often in phage-to-bacteria ratios of 10:1 or greater. As there exist estimates of total bacterial numbers of approximately 10^{30}, there consequently is an expectation that there are 10^{31} or more total individual virus (mostly phage) particles, making phage Earth's most numerous 'organisms'. Bacteria are highly diverse, and perhaps there are millions of species of these prokaryotes. Phage–bacterial interactions therefore are quantitatively vast (huge numbers of interactions) and qualitatively diverse (huge numbers of environment types, bacterial–host types, and also individual phage types). In this article we provide an overview of this enormous diversity of phage types, variations on phage biology, and the importance of phage in the laboratory, in environments, and, potentially, in the clinical setting.

Primer on Phage Biology

F. W. Twort (in 1915) and Felix d'Herelle (in 1917) both described phage as macroscopic phenomena: the dissolving of bacterial colonies, the clearing of turbid broth cultures of bacteria, and the formation of holes (plaques) in turbid 'lawns' of bacteria. Confirmation of phage as viruses awaited the invention of the electron microscope (early 1940s). Subsequently, phage were used in elucidating the basic molecular mechanisms of life, and are credited with significant contribution to the development of the scientific subdisciplines of molecular biology and molecular genetics. In this section we provide an overview of the phage life cycle, which by definition must include at some point an extracellular virion state. We then discuss major criteria by which phage biologists distinguish among different phage genes and different phage types.

Phage Life Cycle

The formation of a free phage in the phage life cycle, which initiates the phage's extracellular search for new bacteria to infect, is discussed first. The extracellular search culminates with adsorption. Entrance of the phage genome into the cell begins the infection period, typically described as the latent period. The latent period ends either with phage-induced bacterial destruction (lysis) or with an extruding or budding of maturing phage virions (chronic release) across the bacterial cell envelope (in a manner that does not lead to immediate bacterial death). Infections that result in phage release can be described as productive. Alternatively, phage infections can enter a latent state (lysogeny or pseudolysogeny) or can fail to successfully infect (phage restriction or abortive infection). See **Table 1** for a summary of phage infection types and their characteristics.

Phage Genes

Functionally, the genes and factors involved in a phage infection can be differentiated into three types: products of bacterial genes, products of phage genes that are expressed and employed during phage infection, and phage genes whose products are associated with released phage virions. Though useful when viewing phage as evolving ecological entities, not all of the gene products employed by phage may be unambiguously differentiated into these three categories. For example, phage virions can carry proteins that are employed only when subsequent infection occurs. Also ambiguously, many phage

Table 1 Different types of phage infections

Type of infection	*Does the phage genome replicate?*	*Are free phage produced?*	*Do bacteria survive?*	*Do phage survive?*	*Comments*
Lytic	Yes	Yes	No	Yes	Productive cycle that releases free phage via phage-induced bacterial lysis, which totally destroys both bacterium and phage infection
Chronic	Yes	Yes	Yes	Yes	Productive cycle that results in the formation of free phage without destroying either the infected bacterium or the phage infection
Lysogenic	Yes	No	Yes	Yes	Phage genome replication without production of phage progeny; replicated genomes segregate into daughter bacteria
Pseudo-lysogenic	No	No	Yes	Yes	Infecting phage genomes do not replicate and do not produce phage progeny; a quiescent state that can lead to productive or lysogenic cycles
Restricted	No	No	Yes	No	Passive or active bacteria-mediated death of infecting phage such as via display of restriction endonucleases
Abortive	No	No	No	No	Abortive infections involve the death of both bacterium and infecting phage without production of phage progeny

Derived from Abedon ST (2008) Phages, ecology, evolution, ST Abedon (ed.), *Bacteriophage Ecology*. Cambridge: Cambridge University Press.

genes encode proteins that are involved in virion assembly but that nevertheless are not represented as part of the resulting virion.

Evolutionarily, phage genes can be distinguished in terms of their potential to coevolve with phage molecules (such as proteins or DNA). Coevolution among phage genes is often cited as an explanation for the genetic linkage – similar location on the genome – of genes encoding interacting phage capsid proteins, such that a mutation in one gene requires a corresponding mutation in another. Alternatively, phage genes can evolve to become further refined in their interactions with smaller molecules (e.g., nucleotides), generic versions of larger molecules (e.g., as with phage exonuclease action on bacterial DNA), or with specific bacterial macromolecules (e.g., RNA polymerase or the phage receptor molecule found on the surface of host bacteria). The latter too can be coevolutionary should antiphage adaptations by bacteria stimulate phage to evade such adaptations.

Phylogenetically, phage genes can be distinguished in terms of where those genes came from. Many or most genes associated with phage, for example, likely have been evolving within phage genomes for millions or even billions of years. Other genes, or portions of genes, appear to be newly acquired from bacterial genomes. Some phage genes seem to readily move from phage genome to phage genome due to genetic recombination following coinfection of individual bacteria by different phage types. Still other genes, such as virion structural genes (below), appear to move between phage genomes only as multigene complexes, presumably as a consequence of the difficulty in splitting these coevolved genes while still retaining function (above).

Metabolically, phage genes may be sorted into at least six broad classes: (1) phage genes involved in the metabolic takeover of host metabolism (e.g., genes that shut down transcription from the bacterial genome), (2) phage genes involved in establishing or maintaining a latent state (e.g., lysogeny), (3) phage genes involved in preparing the infected cell for phage progeny maturation (e.g., genes involved in prophage induction, nucleic acid repair, and nucleic acid replication), (4) genes that express phage virion structural proteins and virion assembly proteins, (5) gene products that process and package newly replicated phage genomes into virions, and (6) gene products required for release of assembled virions from the host cell either by lysing the host cell or by exporting the newly assembled virions through the cell membrane. Though phage genes, as distinct from bacterial genes, were traditionally viewed as models for the basic metabolic workings of life, there is no fundamental biochemical difference between phage and bacteria encoding of parts of complex molecular machines such as those involved in gene transcription.

Genetically, phage genes required for productive infection can be described as encoding essential functions. Mutations in these genes tend to be lethal or effectively so by substantially decreasing the number of progeny produced during infection such that phage plaques do not form. Phage also encode nonessential genes. Inactivation of these genes still allows production of viable progeny. The yield of phage per infection is often reduced, but not to the point that phage propagation is severely curtailed. Alternatively, if the yield is not reduced, and additional phage growth parameters are not otherwise affected, it may be that these nonessential genes encode proteins that are only needed for infection of certain hosts or under certain metabolic conditions. The number of nonessential genes can be significant. Sixteen of the fifty (32%) genes of bacteriophage T7 are nonessential, for example. Finally, it should be noted that some bacteriophage genomes contain other genetic elements such as introns.

Phage Gene Expression

The expression of phage genes typically occurs in a well-defined temporal order, one that typically 'makes sense' in terms of the metabolic function of these genes. In addition, as bacterial parasites, phage rely on the host cell for a range of metabolic functions. In the most genomically complex phage, functions supplied by the host cell may be limited to cell structural elements (membranes, cell wall), energy generation machinery, and protein synthesis apparatus (ribosomes). Large, more complex (and usually obligately lytic) phage often shut down host gene expression soon after entering the cell and may degrade the host genome. Simpler phage usually rely on the host cell for more functions. Temperate and chronically released phage are also more reliant on host cell functions and, presumably as a consequence, are less disruptive of these functions.

Upon entry into the host cell, phage begin expressing what are termed early or immediate-early genes. The promoters of these early genes – DNA sequences where RNA polymerase binds to initiate transcription – tend to more closely resemble bacterial promoters than those of late-expressing phage genes. The early genes of obligately lytic phage produce proteins involved in co-opting bacterial gene expression. These include RNA polymerases and sigma factors (which modify RNA polymerase promoter recognition). Phage-encoded sigma factors favor transcription of viral genes over bacterial genes. Expression of these early genes is followed by delayed-early or middle genes (or, in some cases, late genes) that initiate phage genome replication and related functions. Phage that encode their own DNA or RNA polymerase begin synthesizing that enzyme along with proteins involved in nucleic acid metabolism. Collectively, early and middle genes are sometimes referred to as prereplicative genes, that is, genes expressed before genome replication begins.

The first proteins made by temperate phage are those that determine whether lysogeny or a lytic infection will occur. Expression of these early genes is sensitive to the metabolic state of the bacteria. The relative level of expression of key phage proteins commits the phage to either the lysogenic or the lytic pathway by activating one set of regulatory genes and inhibiting a second set of regulatory genes. If the phage is to enter the lysogenic phase, then proteins that bring about the integration of the phage genome into the host genome or, instead, stabilize the phage genome in an episomal (plasmid) form, are produced. Proteins that prevent (by inhibition or termination) expression from the remainder of the phage's genes are also produced. If the phage enters the lytic cycle, then it begins expressing early and middle lytic genes and blocks expression of lysogenic genes.

During the postreplicative phase of the lytic cycle, both obligately lytic and temperate phage begin expressing late genes, including structural genes for the phage virion, processing proteins that are required to mature the virion (if they encode any), and packaging proteins that place newly replicated phage genomes into the phage particle. Late genes are much more dependent on phage-encoded functions for expression. In some phage, such as phage T7 and T3, late-gene expression is dependent on a phage-encoded RNA polymerase. In other phage, such as phage T4, the switch to late-gene expression is brought about by modification of the *Escherichia coli* RNA polymerase complex by phage proteins. In bacteriophage λ and related phage, expression of late genes begins by a combination of derepression (loss of a repressor protein allowing RNA polymerase access to promoters) and antitermination (caused by a phage protein that allows transcription to pass through transcription termination sites).

A few phage also encode unusual late genes that contribute neither to the phage virion nor to the phage release. Notable examples of this are lambdoid phage strains, which both lysogenize pathogenic *E. coli* strains such as the O157 and encode a type 2 Shiga toxin. In these phage, the toxin genes are located next to the genes causing cell lysis and are expressed with them as late genes. The release of the toxin from the cell is also dependent on phage-induced cell lysis. There is debate as to whether these toxin genes ought to be considered phage genes or, instead, phage-located bacterial genes.

Phage Diversity

Phage, like viruses in general, are typically differentiated in terms of their infection type, virion morphology, or genome characteristics. Based on infection types, a phage may be described as a lytic versus chronic releaser or as obligately lytic versus temperate. Based on virion morphology, phage may be described as tailed (also known as binary), cubic (including icosahedral), helical, or pleomorphic. Tails can be long or short, flexible or rigid, and contractile or noncontractile. Phage genomes can be ssDNA, dsDNA, ssRNA, or dsRNA. Genomes can also be segmented (multipartite) or nonsegmented (monopartite). The majority of phage isolates are tailed and so far as is known all tailed phage display dsDNA, monopartite genomes, and lytic cycles (though many are also temperate). See **Table 2** for a description of the various phage families.

Increasingly, phage are differentiated in terms of their genome nucleotide sequences, and whole-genome sequencing provides a wealth of information useful for inferring phage genomic functionality and evolutionary history. The analysis of phage in this manner is referred to, generally, as the study of phage genomics. Phage additionally may be distinguished in terms of their host range, that is, what bacterial types they are capable of infecting. In general, however, host-range-based classification is less useful in obtaining a facile understanding of common themes of phage biology. Perhaps the best illustration of the difficulty of host-range-based classification are phage P1 and Mu, which are able to switch between different host ranges by utilizing alternate phage-encoded adsorption proteins.

Table 2 General characteristics of major phage types infecting eubacteria

Family	*Genome*	*Morphology*	*Release*	*Examples*
Cystoviridae	dsRNA, segmented	Enveloped	Lytic	ϕ6
Inoviridae	ssDNA, circular	Helical, rod-shaped, or filamentous	Chronic	f1, fd, M13, CTXΦ
Leviviridae	ssRNA	Icosahedral	Lytic	MS2, F2, Qβ
Microviridae	ssDNA, circular	Icosahedral	Lytic	ϕX174
Myoviridae	dsDNA, linear	Tailed, contractile	Lytic	Mu, P1, P2, T2, T4, T6, RB69
Siphoviridae	dsDNA, linear	Tailed, noncontractile, long, flexible or rigid	Lytic	λ, N15, SPP1, T1, T5
Podoviridae	dsDNA, linear	Tailed, noncontractile, short	Lytic	Gh-1, N4, ϕ29, ϕA1122, ϕYe03-12, P22, SP6, T3, T7

Importance of Phage

Bacteriophage play important roles – some positive, some negative, some current, some historical – in biology, ecology, and health. In this section we provide an overview of these roles. These include, in order of presentation, phage use as molecular model systems, phage (and phage product) use as molecular tools (including phage display technologies), phage use in bacterial identification, the problem of phage as contaminants, the promise of phage therapy (i.e., phage as antibacterial agents), phage use as indicators and tracers, the study of phage interaction with animal bodies, the study of phage ecology, phage-mediated transfer of genetic material between bacteria (i.e., transduction), the role of phage in bacterial pathogenesis, and the use of phage models in the general study of organismal ecology and evolutionary biology.

Phage as Molecular Model Organisms

Phage have been used as model organisms for almost as long as their biological nature was recognized (e.g., the use of phage as model viruses by Emory Ellis in the late 1930s). In part this was (and is) due to their ease of culture and to their amenability to chemical as well as biological manipulation. Bacteriophage were used in key experiments in the history of molecular biology including those leading to an understanding of the structure and composition of the gene, mutagenesis, and gene regulation. Two notable examples are a demonstration, by Hershey and Chase, that DNA is the genetic material in phage T2, which served as the second and universally convincing system for which DNA was demonstrated to be the hereditary material, and the demonstration, by Luria and Delbrück, of the existence of mutations within a population before selection, which ushered in the now obvious understanding that bacteria, like peas and fruit flies, also possess an experimentally accessible genetics.

Phage were used in many other key discoveries in the history of molecular biology. A partial list includes recombination of phage chromosomes; viral encoding of metabolic enzymes; restriction/modification enzymes; fine structure of the gene; mechanisms of mutation; existence and function of mRNA; nature of the genetic code; colinearity of genes and proteins; conditional lethal mutations; nonsense and frameshift mutations; protein assembly pathways; role of sigma factors in transcription; *in vitro* gene expression; noneukaryotic introns; and the identification of DNA replication fork proteins.

Phage as Molecular Tools

Just as bacteriophage played a critical role in the development of molecular biology, bacteriophage have been vital in genetic engineering technologies. The use of phage as cloning vectors (carriers of cloned genes), for example, takes advantage of the ability of phage to infect a large population of bacteria essentially simultaneously and begin expressing the cloned gene in a temporally coordinated fashion. This has allowed the expression of proteins that would otherwise be lethal to bacteria. Likewise, the ability of some phage to integrate their DNA into specific sites in the bacterial genome allows for bacterial strain modification that would prove difficult by general recombination.

Many different phage have been used to develop genetic engineering tools and techniques, but three have played especially important roles. These are the temperate phage λ (and the related lambdoid phage), the chronically released filamentous phage M13 (and its cousins f1 and fd), and the obligately lytic phage T7. Phage λ along with the various filamentous phage have been used to develop a series of gene expression and cloning vectors. The organization of the λ genome is especially useful as a cloning vector since the central one-third of the 48 000 bp genome can be deleted, leaving ample room for inserted genes. This segment includes all of the genes responsible for lysogeny, committing the derived phage to a solely lytic life cycle. Phage M13 and T7 have been widely used in phage display technology (below). Functional elements of other phage, including the promoters of phage T3, T7, and SP6 as well as the phage f1 origin of replication, have come to be used in a wide variety of cloning vectors. Filamentous phage (M13, f1) lack a large dispensable region but have other uses since cloning vectors derived from these phage can form a plasmid-like intermediate in the infected cell and replicate and package their genomes in a ssDNA form. Single-stranded DNA is particularly useful for a number of genetic engineering procedures including oligonucleotide-directed site-specific mutagenesis, synthesis of strand-specific probes, subtractive hybridization, and DNA sequencing. Likewise, there are more specialized functional elements, such as the cin recombinase (also called cre recombinase) and recognition sites (*cix-cin* sites) of bacteriophage P1, which have been adapted to make a powerful site-specific recombination system: the Cre/Lox system.

Phage Display

A powerful phage-based genetic engineering tool is phage display. Phage display is a form of high-throughput screening that allows large numbers of peptide segments to be screened for binding to a target. For these systems, a library encoding short peptide segments is inserted into a gene encoding a phage virion capsid protein. Insertion of these peptides is accomplished at a location that leaves the added peptides on the surface of the virion particle, in a manner that minimally disrupts virion functionality, and where they are accessible to interact with other proteins or materials. Depending on the virus vector used, the displayed peptides may be in head proteins, receptor proteins, coat proteins, and so on. The resulting recombinant phage are exposed to a target protein, or other material, that is usually fixed to a surface or other solid matrix. If the inserted protein segment has affinity for the target, then the phage will bind to it. Noninteracting phage are washed away and the bound phage are recovered. The recovered phage are then grown to produce a population enriched for phage whose inserted peptide binds the target. These steps, which are sometimes termed 'biopanning', are repeated through several (commonly three to five) rounds to obtain phage containing the tightest binding segments under the conditions used.

Phage display has been used in finding peptides that bind to a wide variety of targets and that are useful for many purposes including the generation of antibody and other protein-binding peptides (which are useful for epitope mapping and for vaccine development); virus-receptor-binding peptides (which are useful in drug discovery); novel DNA-binding peptides, especially ones that show sequence specificity (which may be employed in designing novel gene regulatory proteins); and peptides that bind to inorganic materials (such as semiconductor materials used to create hybrid organic–inorganic materials). Not all phage display systems work equally well for a given application and finding the most appropriate system for a particular target is sometimes a matter of trial and error.

Phage Use in Bacterial Identification

The treatment of bacterial disease, or otherwise dealing with bacterial contamination (including by quarantining), is eased via rapid and accurate bacterial identification. For decades phage have played important roles in such identification. Early techniques involved phage typing, which is bacterial identification (and classification) on the basis of phage susceptibility. For bacterial detection, phage may be added to bacteria-containing cultures, looking for phage amplification that occurs only if phage-susceptible bacteria are present. Alternatively, phage endogenous to a culture (i.e., naturally occurring) and that infect specific bacteria types may be used to infer bacterial presence. More recently, phage have been tagged with reporter molecules, such as fluorescent markers, or have been bioengineered to express reporter genes, which provide light or color changes within a culture only if phage infection occurs successfully. The latter approach is especially useful in identifying bacterial antibiotic susceptibility since antibiotics capable of blocking phage infection (due to blocks on bacterial metabolism) can also block reporter-gene expression. All of the techniques are dependent in their efficacy on the relatively narrow host range displayed by many phage, that is, the potential by phage to infect only a limited number of bacterial types.

Phage as Contaminants

Biotechnology employs organisms to produce useful products and in the modern world this typically involves employing some form of recombinant DNA. As the use of microorganisms in biotechnology has become more sophisticated, especially beyond their use, for example, as naturally acquired cultures during food production, the tendency has been to employ pure cultures. A pure bacterial culture, however, is especially susceptible to inactivation by contaminating phage. That is, a single bacterial type may be wiped out by a single phage type, whereas wild cultures, consisting of multiple bacterial types, are more likely to contain bacteria that are inherently resistant to any given phage type. With the advent of modern culturing techniques, along with bacterial culturing on industrial scales, phage contamination thus can and often does lead to catastrophic fermentation failure.

Fermentation failures are especially prevalent in the production of fermented dairy products, such as cheeses, since the milk is not sterilized prior to use and therefore can contain phage specific to the starter culture bacteria employed to create the specific characteristics of the fermented food. A variety of steps may be taken to minimize phage contamination, or its impact. These include modifications to fermentation facilities or to the bacterial strains employed.

Indigenous phage have also been implicated in the failure of natural bacterial assemblies. These may include the lactobacilli, vaginal normal flora that otherwise can serve as a bulwark against bacterial vaginosis. It has been hypothesized that cigarette smoking can lead to bacterial vaginosis as a consequence of prophage induction in vaginal lactobacilli lysogens.

Phage Therapy

While phage can be a nuisance because of their ability to infect and thereby inhibit or destroy bacterial cultures of industrial importance, that same propensity can be harnessed to reduce numbers of nuisance bacteria, including bacterial pathogens. This phage application as antibacterial agents is described generally as phage therapy. The history of phage therapy is almost as long as the history of phage study itself, but phage use as antibacterial therapeutic agents was discarded, especially by Western medicine, as a consequence of the discovery of antibiotics. Phage therapy has a number of advantages over traditional antibiotics, however. Phage are self-replicating, minimally toxic, and easily isolated. Furthermore, phage display a 'narrow spectrum of activity' (which for phage is their host range), meaning that only relatively few bacteria are susceptible to a given phage strain. As a consequence, unlike antibiotics with their broader spectrum of activity, phage therapy is much less likely to cause the 'collateral damage' of destroying beneficial bacteria along with pathogenic, target bacteria. Phage therapy therefore is less likely to give rise to side effects such as overgrowth of opportunistic pathogens, so-called superinfections, that sometimes occur such as with antibiotic-associated vaginal yeast infections or *Clostridium difficile*-associated colitis.

Phage as Fecal Indicators and Environmental Tracers

Phage are found associated with fecal material because bacteria are abundant in the animal colon. As feces carry enteric pathogens capable of causing disease in otherwise healthy individuals, public health measures are directed toward both avoiding contaminating water supplies with feces and monitoring water supplies for fecal contamination. In addition to certain bacterial types, phage can be employed as indicators in such monitoring, especially since, as viruses, they can mimic (to a certain degree) the survival characteristics of pathogenic enteric viruses. Also, as mimics of enteric viruses, phage can be employed to test the potential of various water treatments for removal of such viruses, as well as for virus removal, or containment, in a variety of nonwater contexts. Phage are also employed as relatively inert tracers of water movement, including of underground water supplies.

Phage-Based Interactions with Animal Bodies

Phage can be employed as a means of characterizing animal bodies, be it in terms of immune response, their removal from serum by nonspecific immunity, or in mapping tissues using phage display. Phage ϕX174, especially, has been used to study the human humoral immune response, serving as a neoantigen (one not previously experienced by an animal) such that primary, secondary, and tertiary immune responses may be induced simply with subsequent vaccination. Antibody fragments produced by phage display methods can also be used to target specific tissues. A phage display library of antibody fragments is injected into an animal including, in certain instances, into humans. Recovery of phage virions from tissues and subsequent phage growth results in amplification of antibody fragments with specific binding properties and/or a mapping of antibody binding to specific tissues. This can be considered *in vivo* biopanning. Phage that display tissue-targeting peptides may be employed in the course of gene therapy, serving as vectors targeting specific tissues. Here, phage DNA is replaced by appropriate gene therapy vector DNA along with the therapeutic gene of interest. Phage may also serve as platforms for the display of antigens as vaccines. Because phage proteins also provoke an immune response, the phage itself may act as an adjuvant for the displayed peptide, that is, a substance that enhances the immune response.

Phage Ecology

Phage ecology is the study of the interaction of phage with their environments. Environments consist of biotic components (other organisms) or abiotic components (spatial, chemical, and physical factors). Subdisciplines of phage ecology consist of organismal, population, community, and ecosystem ecologies. Organismal phage ecology considers those phage adaptations responsible for phage survival or acquisition of new bacteria to infect. Phage population ecology considers groups of similar phage (loosely, same phage species) that are located within the same environment. Of concern are demographic factors, especially births and deaths, and how these factors are affected by phage and bacterial population densities. Communities consist of more than one species found within the same environment, and phage community ecology considers especially the interaction between phage and bacteria but also among phage or between phage and other organisms that are affected by phage-associated bacterial genes such as toxin genes.

Ecosystems consist of both biotic and abiotic factors contained within a reasonably well-delineated environment, such as a pond or a field, and phage ecosystem ecology considers especially the impact of phage on the movement of energy and nutrients through and within ecosystems. The interruption of such movement by viruses is especially important in aquatic environments, which are principally based on primary production (photosynthesis) performed by microorganisms. In addition, the lysis of these microorganisms provides nutrients to heterotrophic bacteria, which serve as a second base of aquatic ecosystems. Phage disruption of aquatic environments has a significant impact on the global carbon budget and therefore on the global warming that results from atmospheric carbon dioxide accumulation.

Horizontal Gene Transfer

Transduction is the movement of DNA from one bacterium to another via a phage virion carrier that has picked up bacterial DNA during infection. When the virion carrying the bacterial DNA infects another bacterium, the transduced DNA enters the recipient cell following the same entry path normally used by phage DNA. The DNA can then be incorporated into the bacterial genome via recombination. Transduction appears to play an important role in bacterial gene exchange.

In generalized transduction the DNA within a phage virion comes entirely from the bacterial genome.

Generalized transduction can move bacterial genes without distinguishing among them, but is mediated by inviable phage virions. Specialized transduction occurs when prophage excise from a bacterial genome in a manner that incorporates adjacent genetic material. Specialized transducers can retain phage viability, though the likelihood of viability is reduced as the size of the bacterial genetic material transferred is increased. Phage can also incorporate bacterial genetic material via illegitimate recombination, forming what are known as morons (for 'more' DNA). The moron accretion hypothesis posits that phage evolution occurs via the gradual incorporation of morons. Through coevolution with the rest of the phage genome, these morons either are eliminated or come to take on important phage functions.

Phage Role in Bacterial Pathogenesis

As transducers of DNA, phage play important roles in bacterial pathogenesis, the potential of bacteria to cause specific diseases. During lysogeny many prophage can express genes that impact bacterial phenotype, for example, by the production of exotoxins, which are eukaryote-affecting toxic proteins released from the bacterial cell. The expression of these genes is described as either phage conversion or lysogenic conversion. In addition, following induction of a prophage, genes that impact the characteristics of the harboring bacterial culture may also be expressed. Also, via generalized transduction, phage can transfer bacterial DNA as so-called pathogenicity islands, which are contiguous strings of genes that encode factors that contribute to bacterial pathogenicity.

While not all examples of lysogenic conversion contribute to bacterial pathogenesis (superinfection immunity by temperate phage does not, for example), there are many instances where bacterial toxins are encoded by temperate phage either in the lysogenic phase or, less commonly, during the lytic cycle. *Corynebacterium diphtheriae*, for example, is converted from a nonpathogenic form to a pathogenic form after phage infection. Likewise, about 5% of the genomes of pathogenic strains of *Salmonella typhimurium* are composed of prophage genomes, and these prophage contain some of the bacterial arsenal of exotoxin and other virulence factor genes. Pathogenic strains of *Vibrio cholerae* also owe much of their pathogenicity to phage conversion, with cholera toxin encoded by the temperate and filamentous phage CTXΦ. Another variation on this theme is seen in the production of Shiga toxin by pathogenic strains of *E. coli* such as the serotype O157. There are several related Shiga toxins but a typical feature is that their genes are located in prophage genomes and the harboring bacteria lack a mechanism to export the toxin. Instead, Shiga toxin is released when the prophage is induced and subsequently lyses the bacterium to release phage progeny.

Phage as Ecological and Evolutionary Model Organisms

Besides studying phage ecology and evolutionary biology, researchers test ecological and evolutionary biological theories by employing phage. In other words, just as phage have been useful as models for universal molecular aspects of life (e.g., transcription, translation, and DNA replication), they can serve as model organisms for studying universal (or, at least common) aspects of organismal ecology and evolutionary biology. Included are the study of predator–prey interactions, evolutionary optimization of adaptations, the generation of known phylogenies (diverging lineages of evolving populations), genetic constraints on natural selection, and the evolutionary roles played by genetic drift. The advantages associated with employing phage in these studies include their relatively simply biology, their rapid rates of reproduction, the exceedingly high numbers that phage may be grown to, ease of long-term storage, high mutation rates (especially for RNA and ssDNA phage), ease of laboratory manipulation, amenability to genetic engineering (including reverse genetics, i.e., engineering in mutations), and, perhaps especially, the relative smallness of phage genomes (particularly, again, for RNA and ssDNA phage), which allows for routine whole-genome sequencing.

A Closer Look at Certain Aspects of Phage Biology

Certain aspects of phage biology have been extremely well defined molecularly. In this section we provide an introduction to several of these processes, focusing on phage adsorption, virion assembly, lysis, lysogeny, and phage evolution.

Adsorption

Adsorption describes the process by which phage attach to the surface of host bacteria. This process has been especially well defined for the contractile, long tail fiber-containing T-even bacteriophage. The tips of the six long tail fibers bind to specific surface proteins or other bacterial receptor molecules (such as lipopolysaccharide). It has been shown that three of the six tail fibers are sufficient to properly orient the phage onto the surface of the cell, placing the base plate (found at the distal end of the tail) in proximity to the surface. Binding of the long tail fibers is reversible so that if the base plate is not adjacent to its receptor, then the long tail fibers can release. These reversible interactions allow the phage to 'walk' across the surface of the cell until the short tail fibers, found under the phage base plate, are able to successfully bind. Short tail fiber binding is irreversible and, along with the long tail fibers, triggers the opening of the base plate. The remainder of the infection process consists of tail sheath contraction, exposure of phage enzymes that help degrade the cell wall, penetration of the tail tube through the cell envelope to the cell membrane, and subsequent ejection of the phage genome into the bacterial cytoplasm.

Virion Self-Assembly

Assembly of the virion particle is often described as 'self'-assembly because an ordered assembly process in which complex structures are built by the individual component proteins, which self-assemble in a defined order, aided by only a limited number of accessory or helper proteins. In addition, different virion components, such as the head, tail, and, if present, the tail fibers, self-assemble independently and then join together. The tail is perhaps the most complex of the three main components and its assembly illustrates several principles. As outlined in **Figure 1**, in T-even phage, tail assembly begins with the base plate. This is composed of six wedges that are each made up of several different proteins and hub protein complexes. The wedges and hubs combine to form the hexagonal base plate. Once the base plate has assembled, the short tail fibers attach to it. Only after this step is completed does the tail tube form, assembling onto the base plate. Once the tail tube is formed, then the proteins that make up the contractile sheath assemble around the tail tube. Finally, the tail assembly is completed by the addition of connector proteins that will link the completed tail to the head, after its assembly (including genome packaging) is completed. Over 20 different proteins form the tail, but only two chaperone proteins (proteins that aid protein folding) are needed for tail assembly. In each step of assembly, the partially completed structure acts as the initiating complex for the next proteins to bind to and complete their folding to their final form. A similar process is followed by phage heads and tail fibers.

Filamentous phage, such as M13, have a very different assembly pathway that reflects their chronic release. Because of this type of release, completed virions do not accumulate in the infected cell. Instead, they are continually extruded from the infected bacterium and assembly is intimately tied to this extrusion. Assembly takes place at the cell membrane and all phage proteins that are involved in virion formation, including those involved in pore assembly through which virions are extruded, are integral proteins that collect in the membrane. Virion assembly begins when a phage genome, bound to a specific structural protein at the genome packaging site, enters the pore. Additional capsid proteins attach to the growing virion as it passes through the pore until the entire genome is coated with the major coat protein. The capsid is completed with minor coat proteins binding to the terminus, including the receptor proteins. This completion also triggers the release of the now-mature virion.

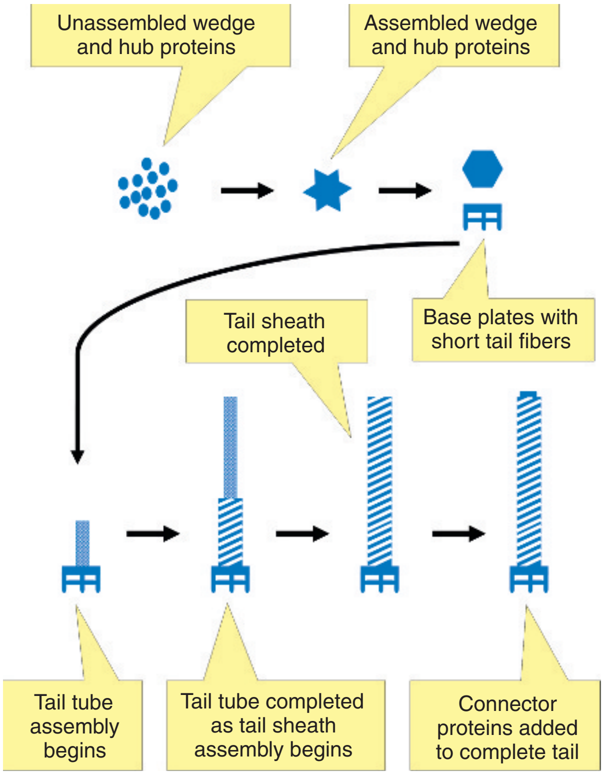
Unassembled wedge and hub proteins
Assembled wedge and hub proteins
Tail sheath completed
Base plates with short tail fibers
Tail tube assembly begins
Tail tube completed as tail sheath assembly begins
Connector proteins added to complete tail

Figure 1 T-even phage tail assembly. Self-assembly of the phage tail begins as wedge and hub proteins join together to form a partial base plate. Base plate assembly is completed by the addition of the short tail fiber and joining proteins. Two completed base plates (top and side views) are shown. The base plate acts as the assembly initiator for the tail tube. As the tail tube assembles, the base plate/tail tube acts as the assembly initiator for the tail sheath. Once the tail tube and, then, the tail sheath are completed, connector proteins are added. The connector proteins will join the head and tail together after the head is filled with DNA. Note that not all steps are shown and that some steps require accessory proteins that are not part of the final structure. For example, short tail fiber assembly requires a chaperonin (a protein necessary for proper folding of another protein into its native conformation). Adapted from Figure 8 of Coombs DH and Arisaka F (1994). T4 Tail Structure and Function. In: Karam J (ed.) *Molecular Biology of Bacteriophage T4*, pp. 259–281. American Society of Microbiology: Washington, DC.

Genome Packaging into Capsids

As part of the self-assembly of phage virions, replicated phage genomes must be packaged into phage capsids. This process has been well studied for the tailed dsDNA phage, which package their DNA into the head portion of the capsid. The process of DNA packaging is not straightforward owing to the length of DNA that must be packaged along with the need, for many phage, to package that DNA into heads at a density that is nearly crystalline. Furthermore, the DNA must be packaged in such a way that DNA exits from heads both smoothly and rapidly. This packaging involves enzymes responsible for moving DNA through ports found in what are known, prior to their maturation, as proheads. All tailed dsDNA phage package their genomes as linear molecules either exactly one genome in length or slightly longer. In both cases the genome is packaged from a large concatemer containing many copies of the phage genome that may be linear or more structurally complex (branched concatemers). Phage that package more than one genome length of DNA package that DNA directly from this concatemer and continue packaging DNA into the prohead until it is full. Hence, it is the capacity of the phage head that determines the amount of DNA that is packaged and the mechanism is described as 'headful' packaging. Because there is more than one genome length of DNA packaged, the ends of the linear molecule are duplicated and this duplicated DNA is often used to circularize the genome after the next infection. Furthermore, this mechanism means that each individual phage packages genomes that have different segments of the genome duplicated, resulting in a genomic map that is circular.

Phage that do not package DNA by headful mechanisms often employ genomic elements that determine the ends of the packaged DNA by defining where the genome is cut from the replication concatemer. Hence, these phage package exactly one genome length and the map of that genome is linear. For example, phage λ flanks the ends of its packaged genome with *cos* sites, which are over 200 bp long and act as recognition sites for the packaging enzymes (terminases) that cleave near the middle of the *cos* sites, leaving a 12 bp overhang. These ends are recognized by other packaging enzymes that place the DNA into the maturing phage head. The *cos* sites also allow for the λ genome to circularize when infecting the next cell. Other phage use similar packaging-enzyme recognition sites that are more generically described as *pac* sites (for packaging).

Lysis

Phage-induced bacterial lysis serves as a means by which mature phage progeny may be released from phage-infected bacteria. Lysis allows rapid release of large numbers of these progeny, but comes at the expense of ending intracellular phage progeny production. That is, lytic phage can continue to produce progeny or release those progeny to find new cells to infect, but not both simultaneously, and only in that order. Phage-induced lysis also has the effect of destroying the infected bacterium, which is relevant with regard to nutrient cycling within ecosystems (i.e., the freeing up of nutrients locked within bacteria as well as the release of intracellular bacterial enzymes, and other molecules, which can go on to modify the extracellular environment). The best studied of phage-associated lysis mechanisms involves at least two components: a protein called a holin and a second protein that digests the bacterial cell wall (the endolysin, also known, generically, as a lysozyme).

The holin controls both the timing of lysis and the access of the endolysin to the cell wall (the cell wall is divided from the cytoplasm, where the endolysin accumulates, by the bacterial plasma membrane). Accumulating in the plasma membrane of the infected bacterium, the holin proteins create holes with precise timing to effect catastrophic exposure of the cell wall to the phage endolysin. For lytic ssDNA phage, in contrast, lysis is achieved via the production and export of a cell wall synthesis inhibitor. As cells continue to grow despite phage replication, these inhibitors eventually cause cell wall failure, giving rise to osmotic lysis of the bacterial cell and thereupon release of intracellular phage progeny. Another type of lysis is lysis from without, which is effected by T4-like phage. In this lysis mechanism, adsorption by multiple phage to a single cell weakens the cell wall, due to lytic enzymes found on the phage tail, contributing to cell wall failure and osmotic lysis of the cell. This mechanism of lysis may serve to augment the lysis by these phage during growth within cultures containing high densities of phage-infected bacteria.

Lysogeny

Temperate phage are capable of causing lysogeny. **Figure 2** outlines the relationship of lysogeny as compared to a lytic infection. The prophage is the temperate phage genome as found during lysogeny. Traditionally, temperate phage are described as integrating into the host genome, and replicating along with the bacterial genome, as seen with the prototype temperate phage, phage λ. However, temperate phage that effect lysogeny by forming plasmids are also known (e.g., phage P1 and N15). Note that temperate phage are not properly described as lysogenic since lysogeny, traditionally, is considered to be a characteristic of bacteria rather than of phage.

Upon infection, temperate phage may be 'reduced' to a prophage rather than display a productive infection. Temperate phage such as phage λ (as well as phage P22 and CTXΦ) display a site-specific reciprocal insertion into the host genome. The steps in this mechanism include a closing of the linear phage genome into a circle, which is followed by recombination over small regions of homology between phage and bacterium at a specific site in the host chromosome. This integration mechanism is catalyzed by the phage integrase enzyme. To go from a lysogenic to a productive state (induction), excision enzymes reverse this integration step. In contrast to phage λ, phage Mu displays a nonreplicative transposition mechanism to insert (rather than recombine) its genome into the host bacterium in a manner that is similar

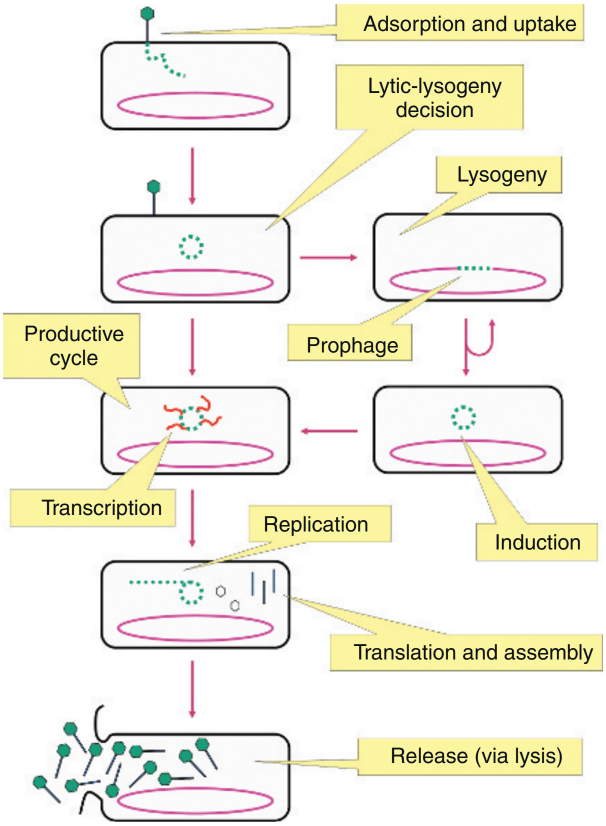

Figure 2 Outline of the life cycles of a temperate phage. After infecting, early proteins made by the phage determine whether the phage will enter the lytic or lysogenic modes. In lysogeny, the phage genome forms a prophage, which for most temperate phage is integrated into the host genome. In this state, bacterial replication also replicates the phage genome, thereby maintaining lysogeny. Some change in bacterial metabolism, typically DNA damage that induces the bacterial SOS pathway, triggers the prophage to enter an active state via the process of prophage induction. The prophage is excised from the bacterial genome and begins expressing phage genes, just as phage that go directly into the lytic phase do. Genes for genome replication as well as virion structural proteins and genome packaging are expressed. The genome is packaged into proheads, virion assembly is completed, and the cell is lysed to release the progeny phage. Note that not all details are shown and that some temperate phage do not follow these specific details.

to that employed by transposons or retroviruses, such as human immunodeficiency virus (HIV). This transposition is not site-specific, unlike phage λ integration, and, as a consequence, results in random mutation by gene disruption of the bacterial chromosome. ‘Mu’ in fact, is named for the first two letters of the word ‘mutation’.

To prevent induction (which is activation of a productive infection), prophage express repressor molecules (usually proteins) that inhibit the transcription of genes necessary for exit from the lysogenic state. In addition to maintaining lysogeny, the repressor protein is responsible for causing immunity (superinfection immunity), which is the inhibition of infection by phage of the same type. For many temperate phage, induction occurs following DNA damage, which induces the bacterial SOS system, leading to repressor inactivation by cleavage.

Mosaic Model of Phage Evolution

The earliest studies on the arrangement of genes in phage genomes showed that these genes tend to cluster by function. For example, most of the virion head protein genes tend to be adjacent to each other within the genome and may even express as a single cistron. This is advantageous as it coregulates genes of similar function and also tends to increase the genetic linkage of genes of related function. As more and more phage genomes have been sequenced, it has become clear that genes of related function are often exchanged in blocks via recombination events. This recombination can be illegitimate, thereby involving no sequence homology among the regions swapped. Alternatively it may involve minimal homology. In addition to the swapping of gene blocks, individual genes, or even parts of genes, may be swapped as well.

These nonhomologous or minimally homologous exchanges are probably fairly rare, and most recombination events likely result in phage genomes that are fatally defective. Large numbers of phage and phage infections presumably allow for sufficient recombination, however, between divergent coinfecting phage such that gene exchange despite minimal homology is a major mode of evolution for bacteriophage. These exchanges should occur especially during productive phage infections of bacterial lysogens, with recombination occurring between infecting phage and prophage, or among prophage present within a single bacterial cell. The result is that phage genomes typically are mosaic, with different genes or regions found within a given phage genome displaying greatest homology with a diversity of different phage. Homology is also found with bacterial genes but, in many cases, phage genes with no homology to previous gene sequences are found, suggesting that there exists an enormous diversity of genetic information encoded by phage from all over the world. In addition, regions of genome homology between phage that otherwise are quite distinctive (e.g., phage T4 and phage λ) are not uncommon.

Overview of Well-Studied Phage Types

A number of phage types have been extensively studied and serve as models for understanding phage biology. Among these phage are the famous ‘T’ (for type) phage of *E. coli* B, which were assembled by Milislav Demerec with the help of Max Delbrück: phage T1 through T7. We present an overview of these T phage first, starting with phage T4, the best studied among them. We then discuss phage λ, which is also a coliphage and which serves as the archetypical temperate phage. Then we turn to brief synopses of a number of additional phage types.

Phage T4 (also T2, T6, RB69, and others)

Bacteriophage T4 is a well-studied, obligately lytic phage of *E. coli*. It is a member of the family Myoviridae, meaning that it has a contractile tail as well as a large icosahedral head. Phage T4 has a relatively large dsDNA genome of approximately 170 000 bases, including modified bases (glycosylated hydroxymethylcytosines) that allow the phage genome to resist digestion by numerous bacterial restriction endonucleases. As one of the best studied of phage types, the biology of phage T4, like that of phage λ, may be considered archetypical. In studies of phage evolution, phage T4 along with phage T2 and T6 form the original members of the T-even family of bacteriophage, which now includes many members that infect many other species of bacteria besides *E. coli*.

Among the interesting, additional characteristics of phage T4 biology are a highly structured self-assembly pathway for virion particles, multiple modes of DNA replication initiation that are utilized at defined times during the replication cycle, a gradual subversion of the host RNA polymerase complex by a combination of covalent modification and replacement of accessory proteins that allows sequential recognition of various classes of phage promoters, gradual breakdown of the host genome and mRNA to supply nucleotides for phage replication and transcription, a large number of nonessential gene products, unusual genetic features including overlapping genes, and the first noneukaryotic introns identified.

Phage T1 (also TLS and others)

Bacteriophage T1 is another member of the classic seven T phage. It is also an obligately lytic virus of *E. coli*, but unlike T4 it is a member of the Siphoviridae family, having an icosahedral head and a long, flexible, noncontractile tail. The T1 genome consists of a single dsDNA molecule of just under 50 000 bp. Phage T1 and related phage are less well studied than the T-even family, in part due to T1's early reputation as a troublesome phage: T1 virion particles are notoriously durable and persistent, resistant to desiccation, and able to spread as aerosolized particles, resulting in its reputation as a contaminant. Stories of labs requiring periodic sterilization or hanging ultraviolet lamps abound, as does the (likely apocryphal) story of an early researcher recovering a sample of T1 from the letter sent by a colleague declining to send a sample. While some of these stories may be exaggerated, it is true that many commonly used laboratory strains of *E. coli* carry the *tonA* or *tonB* allele, which confers resistance to phage T1 infection.

Phage T7 and T3

Morphologically, T7 and T3 are members of family Podoviridae, possessing an icosahedral capsid with a short tail and tail fibers. The T7 genome is just under 40 000 bp of dsDNA. Unlike most phage, the packaged genome only occupies about half of the virion head. The remainder of the space is taken up by a large complex of internal proteins that the genome is coiled around. Packaging of the phage genome is less precise than with other phage, and DNA of between 85% and 103% of the full genome may be packaged. The internal proteins are ejected from the phage head before genome ejection and seem to form a channel through the cell membrane for the genome. Genome entry into the cell is unusually slow for bacteriophage and seems to require active transcription of the initially entering DNA (850 bp) for the remaining genome to enter the cell. It appears that it is transcription itself rather than the expression of particular genes that is the key to phage DNA internalization.

Both phage T7 and T3 produce their own RNA polymerase for late-gene expression. These RNA polymerases are unusually specific for the phage promoters and have found widespread use in many commercialized gene expression vectors. Phage T7 has also found use as a phage display vehicle.

Phage T7 and T3 are two members of a larger group of phage related by genomic analysis. Not all members are coliphage but include, for example, phage infecting *Yersinia*, ϕA1122 and ϕYeO3-12, and a *Pseudomonas putida* phage, gh-1. These other phage display high degrees of homology to phage T7 genes, as consistent with the mosaic model for phage evolution. The host range of phage T7 supports this family linkage: Wild-type T7 can infect some species of *Salmonella* and *Shigella*, but extended host range mutants that can infect some *Yersinia* species have also been isolated as have some mutants of ϕA1122 that can infect *E. coli*.

Phage T5

Bacteriophage T5 is an obligately lytic phage of *E. coli*, which is classified as a member of the Siphoviridae family, having an icosahedral head and a long, noncontractile tail. Its genome is composed of a single linear piece of dsDNA, with just over 121 000 bp. Although its life cycle has several unusual features, phage T5 is not nearly as well studied as other phage types considered here. In part this lack of study is due to the presence of a large number of unusually strong promoters that interfere with bacterial metabolism when cloning of genomic fragments is attempted. Among the unusual features are a number of nicks in the packaged genomic DNA, whose function is unknown, and the excretion of nucleotides from the infected cell soon after infection. This excretion ends after the genome has completely entered the cell.

The entry of the phage genome into the cell is also different from most other families of phage in that phage T5 (and a few near relatives) do not eject their entire genomes into the cell at one time. Instead, about 8% of the genome is ejected into the cell. Expression of the genes on this segment is necessary for the remainder of the genome to enter the cell. Ten proteins appear to be expressed from this genome segment, although not all are essential for genome entry. The pause between the first-stage and the second-stage genome transfer is about 5 min.

Phage λ

Phage λ displays the Siphoviridae morphology, possessing an icosahedral head and a long, flexible, noncontractile tail. Phage λ is the type phage for a large number of related phage usually described as lambdoid and though phage λ infects *E. coli*, other lambdoid phage infect strains of *Salmonella*, *Shigella*, *Pseudomonas*, *Burkholderia*, and so on. Though strictly speaking the term lambdoid is reserved for those phage that can form recombinants with phage λ when they enter the same cell, the term is increasingly being used more loosely to describe any phage that has the appropriate morphology and a temperate life cycle.

The genome of phage λ is a single dsDNA molecule about 48 500 bp long. In its intracellular circular form, the genome can be readily divided into a single portion of about one-third of the genome that contains genes related to lysogeny. Another segment constituting the other two-thirds of the genome contains structural and other lytic-phase-related genes. When the genome is packaged, the lytic phase genes are divided, forming right and left arms of the genome flanking the central lysogenic segment. The temperate life cycle and lysogenic state are described elsewhere in this article.

Phage N4

Phage N4 is a member of the family Podoviridae, which infects a limited number of *E. coli* K-12 strains. Unlike other coliphage, which infect using receptors that are fairly common on the *E. coli* surface, there appear to be only five attachment sites for N4 on each cell. The N4 genome is a single linear dsDNA molecule of about 70 000 bp. The left end of the genome is unusual in that it has a 5–6 base 3′ overhang while the right end varies between substrains and may be blunt or have a 1, 2, or 3 bp 3′ overhang.

The virion of phage N4 is also unusual, not in its structure, but in its contents. In addition to the phage genome, there are one or two copies of a phage-encoded RNA polymerase, designated vRNAP. This RNA polymerase is essential for phage N4 early gene transcription. One of those early transcripts is for a second RNA polymerase, RNAPII, which mediates middle gene transcription. Surprisingly, phage N4 late-gene transcription appears to be effected by the host *E. coli* RNA polymerase. Even more surprisingly, the phage-encoded ssDNA-binding protein, normally employed during DNA replication, is also needed for transcription from late genes.

Phage ϕ29

Phage ϕ29 and related phage are Podoviridae that infect Gram-positive *Bacillus* species, especially *Bacillus subtilis*, as well as other, especially Gram-positive, bacteria. These phage are notable for their relatively small genomes, which are linear, dsDNA, and about 20 000 bp long. Phage ϕ29 has served as a model system for the study of transcriptional regulation, DNA replication, and virion morphogenesis.

Phage SPP1

Phage SPP1 is a well-characterized generalized transducing phage of *B. subtilis* possessing a Siphoviridae virion morphology. It has a linear dsDNA genome of about 44 000 bp. Phage SPP1 and related phage are capable of effecting the transduction of plasmids. SPP1 has also been employed to study phage transfection, which is the transformation of competent bacteria with a phage genome leading to infection.

Phage P1

Phage P1 is a temperate phage of the Myoviridae family, possessing an icosahedral head and a long, contractile tail. Phage P1 can mediate generalized transduction between *E. coli* strains and other Gram-negative bacteria. In addition to its generalized transducing abilities, phage P1 has two other features that are unusual among bacteriophage: First, though a temperate phage, P1 does not integrate its dsDNA genome into the bacterial genome. Instead, the genome circularizes and replicates in a plasmid-like state. The phage devotes several genes to controlling replication and partitioning of the plasmid so that it is stably maintained at low copy number.

Second, phage P1 also has two complete sets of tail fiber genes, each conferring a different host range. These genes are arranged in opposite orientation in the phage genome with a single control region located in between. A phage-encoded inversion system switches the control region from one orientation to the other, activating one or the other set of tail fiber genes. Any single burst contains identical progeny. However, during the lysogenic phase the inversion system is active enough so that a population of lysogens produce about 50% of phage of each host range type. It is this inversion system that has been adapted to create the Cre/Lox recombination system.

Phage P2

Phage P2, like phage P1, is a temperate Myoviridae of *E. coli* and, in fact, both phage were isolated at the same time. The phage P2 genome is a single, linear dsDNA molecule, about 33 000 bp in length. Unlike phage P1, phage P2 integrates its genome into the host genome during lysogeny and is not particularly efficient at generalized transduction. Phage P2 and related phage, however, do seem readily able to capture nonessential genes that have no role in phage metabolism – morons (as described above) – that allow the phage to confer lysogenic conversion phenotypes to host bacteria. In addition, these related phage display extensive mosaicism similar to that of the T-even and lambdoid families of phage.

Phage P4

Phage P4 is a satellite phage, only able to productively infect *E. coli* strains that are P2 or related phage lysogens. P4 is dependent on the P2 genome for all of its virion proteins. Consequently, morphogenetically, phage P4 is also a Myoviridae, although a P4-encoded protein causes the heads to be smaller than normal phage P2 heads. This smaller head size reflects the smaller genome size of phage P4. The linear dsDNA genome is only 11 600 bp compared to the over 33 000 bp of phage P2.

Phage P4 is also a temperate phage and can form *E. coli* lysogens irrespective of the presence or absence of a phage P2 genome. The P4 genome can exist in the lysogenic state as a multicopy plasmid form or it can integrate into the host genome at a specific integration site similar to phage λ. Because of this dual mode of lysogeny, the genome is sometimes described as a phasmid (phage plasmid). Alternatively, the phage P4 genome is described by some as a plasmid that uses an especially effective means of transferring between cells: transduction by phage P2. This description also reflects genetic analysis of the phage P4 genome, which indicates that it is not merely a defective form of phage P2 but rather an independently arising phage. It should also be noted that phage P4 is not unique as a satellite phage, although the number of other examples is still very small.

Phage N15

Phage N15 is a temperate phage displaying Siphoviridae morphology of an icosahedral head and a long noncontractile tail. It is quite similar to phage λ in genome size and organization as well. It deviates from the lambdoid life cycle in the lysogenic phase, where the phage genome does not integrate into the host genome or form a circular, plasmid-like form. Instead, it remains as a linear episomal element separate from the host genome. Phage N15 is one of only three known examples of phage that are maintained as linear plasmids during lysogeny and the only one to do so in *E. coli*. It does this by forming a circular intermediate upon entering the cell that is acted upon by a phage-encoded protelomerase that cuts the circular genome and covalently closes each end of the molecule by joining the two strands together. Replication is accomplished in several modes, which either involve cutting of one or both of the ends for replication and resealing or lead to the production of a circular dimer that is cut and resealed as two linear closed-monomer genomes.

Phage P22

Phage P22 is a temperate phage of *Salmonella* that is often described as lambdoid because of its temperate life cycle and similar genetic structure. It differs from λ in that phage P22 is a member of the family Podoviridae, with adsorption organelles, the tail spikes, attached directly to one of the vertices of the phage particle. Also, while λ is a specialized transducing phage, P22 is a generalized transducing phage. Phage P22 and related phage are considered to play an important role in the evolution of pathogenic strains of *Salmonella*, and many P22-like phage have acquired genes that make lysogens more virulent through phage conversion.

Phage Mu

Phage Mu is a member of the family Myoviridae, with an icosahedral head and contractile tail. Its genome, packaged, consists of a single, linear DNA molecule with about 37 000 bp. In addition, the phage usually packages between 500 and 3000 bp of host DNA, making phage Mu a generalized transducing phage. Phage Mu exhibits a temperate life cycle with genome integrated into the chromosome of its host *E. coli*. It is after integration that the Mu genome displays an unusual alternate mode of movement, acting as a transposon that moves from one location in the host genome to another. It can do this in a nonreplicative manner, leaving one location and inserting into another, or it can utilize a replicative transfer mode and a second copy of the genome/transposon is then created at the second location. Phage Mu is not unique in this dual existence. Mu-like prophage have been found in other bacteria including *Pseudomonas*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Deinococcus radiodurans*.

In addition to its dual prophage/transposon nature, phage Mu has other notable features including a dual set of tail fiber genes in a manner similar to phage P1. Each set confers a very different host range: *E. coli* and *Salmonella* strains in one mode and *Shigella*, *Enterobacter*, *Erwinia*, and *Citrobacter* strains in the other. Finally, as a transposon, Mu has been used to modify and study a wide variety of bacterial genomes, many of which phage Mu could not infect. These range as far from *E. coli* as strains of *Rhizobium* and *Agrobacterium*.

Phage Associated with Different Bacterial Hosts

Archaeal Viruses

The Archaea represent one of the three cellular domains of life, the others being the bacteria and the eukaryotes. Typically, researchers prefer to not describe the viruses of this lineage as phage, though archaeans are bacteria-like in the sense that the cells of both domains lack cell nuclei. Many archaeal viruses, however, are tailed (just as many or most bacteriophage are tailed), though among archaeal phage are also a number of morphologies that are quite unlike those found among bacteriophage (**Figure 3**). Presumably, archaeal viruses play roles in archaean ecology similar to what bacteriophage play in bacterial ecology. In addition, there appears to be evolutionary kinship between certain archaeal viruses and bacteriophage.

Phage Found in Marine Environments

Marine phage are notable especially in terms of their great numbers and probable impact on marine bacteria. Recently a wealth of information has accumulated on these phage, an exploration that is driven, to a great extent, by the potential impact marine phage have on global carbon cycles as important converters of bacterial biomass into dissolved organic matter. To some degree the phrase marine 'phage' is a misnomer, however, as direct counts of virus particles, as marine viruses are often observed, consist not just of bacteriophage but also of archaeal as well as eukaryotic viruses.

Cyanophage

Among marine as well as freshwater bacteriophage are those infecting cyanobacteria, once described as blue-green algae. Cyanobacteria are bacteria possessing a plant-like photosynthetic apparatus and are responsible for generating a significant proportion of the Earth's atmosphere oxygen. They are also important producers in aquatic systems (i.e., photosynthetic fixers of CO_2). Consequently, the lysis of these bacteria by phage (i.e., by cyanophage) redirects much of the resulting organic carbon (and energy) from

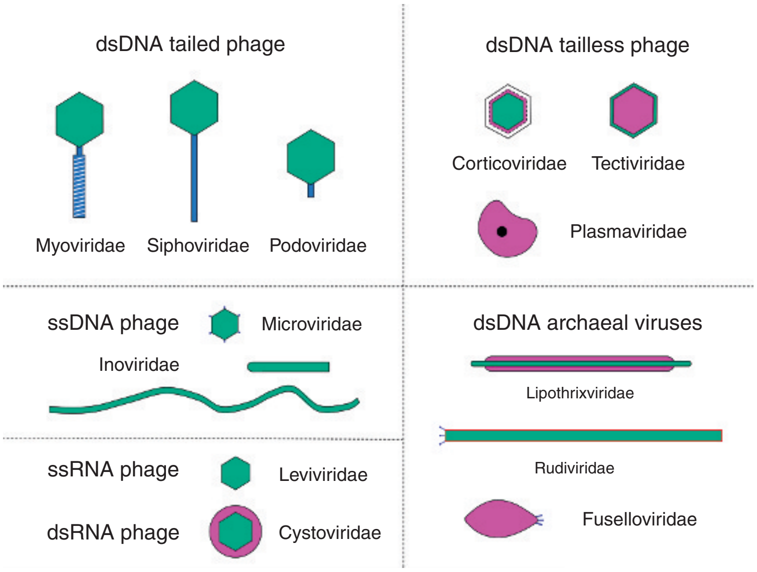

Figure 3 Basic morphologies of different families of virions of prokaryotes. Green represents nucleic acid-encasing capsid protein. This includes the heads of tailed phage. Blue indicates tails and other adsorption organelles (tail fibers and other appendages on tailed phage, however, are not shown). Pink is lipid of various kinds. The contractile portion of the Myoviridae tail is indicated with diagonal lines. The flexibility often seen with the Siphoviridae tail is not indicated. All the heads shown are isometric, but in addition, tailed phage often display elongated (prolate) heads. The Inoviridae are depicted as two types: short rigid rods (genus *Inovirus*) and long flexible rods (genus *Plectovirus*). Long rigid rods of genus *Plectovirus* also exist. Plasmaviridae are unusual budding viruses of mycoplasma bacteria, which lack cell walls. Not all archaeal virus morphologies are depicted. Reproduced from Ackermann H-W (2006). Bacteriophage classification. In: R Calendar and ST Abedon (eds.), *The Bacteriophages*, 2nd edn. pp. 8–16. Oxford University Press.

especially the protist predators of cyanobacteria and redirects it into dissolved organic matter. Dissolved organic matter is mostly unavailable to eukaryotes and nourishes heterotrophic bacteria (themselves prey of phagotrophic eukaryotes as well as of phage). Characterization of cyanophage of the genera *Synechococcus* and *Prochlorococcus* has been especially productive. Among the interesting observations of cyanophage is the phage encoding of proteins that play roles in host cell photosynthesis during infection.

Lactobacillus Phage

The Gram-positive *Lactobacillus* species serve as the basis for the production of numerous fermented food products such as yogurt or cheeses. Because of their economic importance and because phage contamination can give rise to failures in *Lactobacillus* ferments, much of the research concerning *Lactobacillus* phage, and lactobacilli, has been dedicated to devising means of effecting phage resistance. Phage-based technologies, though, may also be employed to enhance food production such as by better controlling *Lactobacillus* lysing to accelerate product ‘ripening’. Comparative genomics using *Lactobacillus* phage is an important area of phage research. This importance is due, in part, to the numerous sequences of *Lactobacillus* prophage that have become available as economically important *Lactobacillus* species have been sequenced, but also because of the economic importance of *Lactobacillus* phage themselves.

Lactococcus Phage

Members of genus *Lactococcus*, like lactobacilli, are Gram-positive, lactic acid-producing bacteria, though unlike the lactobacilli, which are facultative anaerobes, lactococci are aerotolerant anaerobes. Of the lactococci, by far the most economically important species is *Lactococcus lactis*. Because *L. lactis* is employed in large, nonsterile industrial ferments, phage-associated fermentation failure is an economically important concern, especially as a limited number of starter culture bacteria strains have come to be employed per ferment.

Listeria Phage

Listeria monocytogenes is a Gram-positive foodborne pathogen. *Listeria* phage have been used as phage typing agents and more recently have been developed as reporter phage for detection of *Listeria* contamination especially in foods. While most *Listeria* phage are productive at temperatures ranging from 10 °C to 37 °C, some isolates of *Listeria* phage are productive only at 25 °C, suggesting a niche that does not include warm-blooded *Listeria* hosts.

Mycobacterium Phage

The acid-fast mycobacteria are important disease agents (e.g., tuberculosis) but nonpathogenic mycobacteria are commonly found as soil saprobes. Mycophage (the phage of mycobacteria) are important for their role as molecular tools useful in the study of mycobacteria, especially their genetics. As an offshoot of those efforts, mycobacteriophage serve as a phage cohort that is employed in the study of comparative phage genomics, the study of entire phage genomes. An interesting clinical role for mycophage is in phage-based bacterial detection schemes including ones that assess antibiotic susceptibility.

Mycoplasma Phage

Mycoplasma consist of a number of genera of cell-wall-less bacteria evolutionarily derived from Gram-positive bacteria. Due to the lack of a cell wall, mycoplasma phage adsorption is more like that of eukaryotic viruses than that of more typical bacteriophage. Not unexpectedly, then, some mycoplasma phage are enveloped, though others display short tails. In addition, like many enveloped animal viruses, included among mycoplasma phage are ones that are persistently released by budding rather than the extrusion seen with filamentous phage.

Streptomyces Phage

Streptomyces, important producers of antibiotics, are common Gram-positive bacteria in moist soil where they superficially resemble fungi in terms of their mycelial growth as well as their production of drought-resistant spores on aerial hyphae. *Streptomyces* phage have played important roles as tools in *Streptomyces* genetic engineering.

Yersinia Phage

Yersinia is a genus of Gram-negative bacteria that includes, among others, the causative agent of plague. *Yersinia* phage have been isolated primarily for use in phage typing.

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Biofilms, Microbial

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Defining Statement
Biofilm Structure and Function
Biofilms in Natural Ecosystems
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Biofilms in Medical Systems
Postlude
Summary
Further Reading

Glossary

genotype Genes possessed by organism.
phenotype Genes expressed by organism.
planktonic Free floating.
sessile Stationary.

Abbreviations

ENT	ear nose and throat
FISH	fluorescent *in situ* hybridization
SRB	sulfate-reducing organisms

Defining Statement

Biofilms are surface-associated bacterial communities that predominate in natural and pathogenic ecosystems. The matrix-enclosed bacterial cells in these communities assume a phenotype that differs profoundly from that of their planktonic counterparts, and this mode of growth protects them from so many antibacterial factors that they constitute protected enclaves in hostile environments and chronic infections.

Biofilm Structure and Function

Direct observations of a very large number of natural and pathogenic ecosystems have shown that the vast majority of bacteria in these systems live in matrix-enclosed communities attached to surfaces. The term 'biofilm' was coined to describe these sessile communities, and the definition of this term has been refined to be "a community of bacterial cells enclosed in a matrix, at least partially of its own production, which functions as a physiologically integrated community." Further examinations, using the methods of modern microbial ecology, have shown that most of the cells in biofilms are alive, and that cells of different species are often spatially arranged in patterns that facilitate metabolic cooperation. The discovery that sessile cells in biofilms express genes that are profoundly different from those expressed by their planktonic (floating) counterparts led us to describe the biofilm phenotype, and the realization that these patterns of gene expression differ by as much as 70% offers at least a partial explanation of the enormous survival value of biofilm formation. A biofilm is not simply a slime-enclosed mass of planktonic cells. A biofilm is a multicellular community in which each sessile cell expresses its genes in different ways, and this biofilm phenotype is profoundly different from that of planktonic cells in a single-species culture.

We have proposed that biofilms have predominated in all natural ecosystems, from the earliest of times, because these sessile communities provide protection from biological predators and antibacterial chemicals, and because biofilm formation anchors these communities in favorable locations. The life of the planktonic cells shed from biofilms, in the primitive earth, would be 'nasty and short' (Oscar Wilde), except in rare instances in which they discovered virginal surfaces in halcyon environments, but the biofilm from which they set forth would remain protected and inviolate. This distinction is particularly true in pathogenic ecosystems in which planktonic cells are killed by antibodies produced in response to modern vaccines and by antibiotics, while any biofilm that can become established in the body persists for years in myriad chronic infections. Cells within biofilms in chronic infections persist for years, in spite of the focused attacks of many functional host defense systems, and biofilms in natural ecosystems withstand heavy grazing pressure from protozoa and from specialized predators such as snails. We are just beginning to discover the mechanisms of this protection, which may involve compounds that paralyze phagocytes and poison grazing protozoa, but

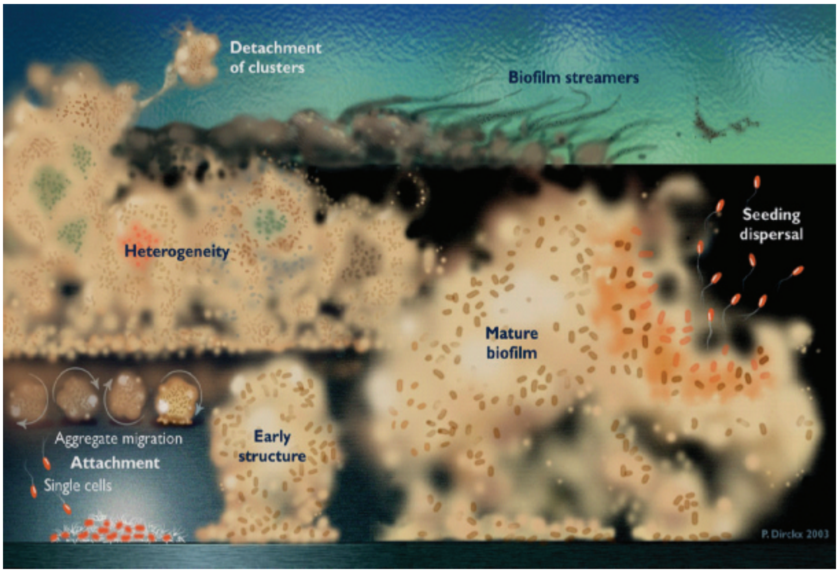

Figure 1 Diagrammatic representation of a microbial biofilm showing the development of these communities, from the attachment of planktonic cells to the development of complex mature microcolonies with open water channels and hollow areas from which planktonic cells have dispersed. The metabolic integration of biofilms is implied, in the middle distance, by the juxtaposition of clusters of physiologically different organisms in arrangements that would facilitate interactions. In the far distance, the diagram suggests a structure somewhat like a kelp bed, in which individual microcolonies are anchored on a surface, but are free to respond elastically to shear forces operative in the environment.

the simple fact remains that biofilms persist and predominate in virtually all ecosystems. The 'life cycle' of biofilms is diagrammed in **Figure 1**.

The complexity of the architecture of mature microcolonies, like that in the right foreground in **Figure 1**, suggested that the process of biofilm formation must be under the control of some kind of signaling system. Many researchers have now discovered which of the several cell–cell signaling systems control biofilm formation in many bacterial species; one 'master system' controlling cell detachment has also been discovered by David Davies. This major discovery reveals that the sessile bacteria in biofilms are sentient of the presence of neighboring organisms, and of environmental conditions, so the groundwork is laid for the concept of biofilms as multicellular communities whose component cells can communicate with each other. This logical process could lead to a situation in which a stimulus applied to one part of a biofilm could trigger reactions that would ramify throughout the community, and produce reactions in locations far distant from the stimulated area. Preliminary indications of this ability to sense changes, and to react on a community basis, have been seen in bacterial biofilms.

If we look at the structural complexity of biofilms, and their behavioral characteristics, we become motivated to discover the mechanisms that enable this complexity and these reactions. When we see that sessile cells in energy-deprived areas of biofilms can receive energy from more favored areas, we discover electrically conductive nanowires that can be used for energy sharing and may even be used for electrical communication. When we note that the frequency of horizontal gene transfer in biofilms exceeds that in planktonic cell suspensions, we find large numbers of pili that may facilitate conjugation, and may also position each cell in a predetermined location in the community. When we note that some *de facto* biofilms (e.g., myxobacterial swarms) move through soil containing stationary biofilms of thousands of species, without losing contact with each other, we note that these cells produce large numbers of signal-containing vesicles that may be 'addressed' exclusively to cells of the same species. The notion of biofilms as sophisticated and integrated communities is new, and we are just beginning to find the first few mechanisms that enable this sophistication and this integration. So we are well advised to marvel at the biofilm's accomplishments, and to ferret out the mechanisms that enable each marvelous attribute, until the whole complex-integrated apparatus is revealed.

The revelation that biofilms predominate, in virtually all natural and pathogenic ecosystems, must drive immediate changes in the ways that microbiologists study bacteria. The reductionist approach of studying a single 'type' strain, growing as planktonic cells in a defined liquid medium, may reveal the arcane secrets of protein synthesis but it has nothing at all to do with bacterial processes in nature or disease. When we select a type strain we make an arbitrary choice, from a spectrum of genomes that comprise the supergenome of the species concerned, and we exclude as many as 1500 genes from consideration. When we study gene expression using a chip made from a type strain, we are blinded from any information concerning the thousands of genes in the supergenome that are not in the type strain. When we study planktonic cells, we study a phenotype that may vary from the biofilm phenotypes that grow in natural and pathogenic systems by as much as 70%, in terms of the genes that are expressed. We can use mutants to study metabolic processes in planktonic cells, and add more exquisite details to the well-worn cycles that are memorized in Microbiology 101, but we cannot use cultures to study mutations that affect the fitness of an individual cell to function as a member of an integrated community.

Modern microbial ecology has long since abandoned the general practice of extrapolating from cultures to ecosystems, and the other subdivisions of microbiology must soon follow. Ecologists analyze ecosystems of interest by harvesting bacterial DNA and sequencing the 16S rRNA gene to determine which species are present. Fluorescent *in situ* hybridization (FISH) probes are then constructed, so that individual cells of the species concerned can be identified in microscopic examinations of the real community growing in the ecosystem. These probes also reveal the distribution of cells of each species, in spatial relationships to those of other species, and to the surface on which the biofilm has formed. The whole community can be studied, intact and in its real surrounding, and the effects of various stresses on the biofilm can be assessed using parameters such as carbon fixation or the output of specific products (e.g., organic acids). The somewhat draconian parameter of cell death can be assessed, *in situ* in real ecosystems, using the live/dead BacLite probe and the confocal microscope. The parallel systems of microscopy-based and nucleic acid-based ecological methods have recently been joined, by the PALM 'capture' microscope and the MDA amplification system, which allows us to visualize a group of bacteria in a real biofilm and then to excise those cells, extract their DNA, and sequence their genome.

Because biofilms predominate in virtually all natural and pathogenic ecosystems, serious students of bacteria must examine the biofilm phenotype of their minute subjects. In the rare instances in which a biofilm is formed by single species, as in certain device-related infections, a single-species biofilm can be grown on an inert surface in the laboratory and valid extrapolations from the culture to the infection may be made. The mixed species biofilms that predominate in most ecosystems are much more difficult to study in the laboratory, even though some simulations may be useful, and direct observations will provide the best data. As we undertake new large-scale projects, such as the NIH roadmap project on the Human Microbiome, we will turn to direct examination of nucleic acids for population analysis and direct confocal and electron microscopy for community mapping, and microbiology will have moved on to a new phase.

Biofilms in Natural Ecosystems

Perhaps because of the historical tendency of microbiologists to 'sample' natural ecosystems, and to head straight for the laboratory with these samples, the bulk of the microbiology of natural ecosystems has involved 'grab' samples of the bulk water phase. When we have profiled natural ecosystems including streams, lakes, and near-shore marine and subsurface environments, we have observed that >99.9% of the bacteria grow in biofilms adherent to surfaces, and only a few stray planktonic cells inhabit the bulk water phase. For this reason, complex organic compounds placed in contact with samples of the bulk water of such systems as the Athabasca River, in the region of the tar sands, show only very slow rates of bacterial degradation. When the complex organics (e.g., bitumen) are placed in contact with the enormous biofilm populations, which have developed in response to the continuous availability of these energy-rich substrates, their degradation is very rapid and complete. The validity of these analyses is attested by the fact that the tons of bitumen washed into the Athabasca River by erosion are completely biodegraded by the time the river reaches Lake Chippewa (48 miles downstream), and by the fact that local oil spills are resolved very rapidly by the biofilm system of the river. If we take a rational view of the microbiology of natural ecosystems, based on direct observations of the location and activity of all of the bacteria, we can assess the real potential of each system for the processing of organic molecules, including pollutants. Our analyses of the Peace/Athabasa/MacKenzie river system of Northern Canada suggest that that this system could be designated as an 'oil-adapted' corridor through which oil from that region, and oil from the North Slope deposits in Alaska, could be shipped with complete ecological impunity. The omission of biofilm populations from ecological evaluations, including impact statements, is bad science and very bad public policy.

The addition of the biofilm component has solved many ecological mysteries that have led classically inclined microbiologists to throw up their hands, and to declare that the ways of bacteria are simply 'wondrous strange'. The puzzle concerning the bovine rumen arose because a fastidiously anaerobic bacterial population, dependent on ammonia, grew and functioned happily in an animal organ that was continuously perfused with oxygenated blood containing large amounts of urea. Then we discovered a special biofilm population on the rumen wall, which developed in the first few days of life, and both scavenged oxygen at the tissue surface and changed urea to ammonia, while deriving energy from the proteolytic degradation of shed epithelial cells. Rumen ecologists also grappled with the problem of laboratory model systems in which biofilms of primary cellulose degrading bacteria broke down cellulose at rates 100-fold slower than the rates seen in the functioning rumen, until they discovered that elusive treponema cells could enter the biofilms for short excursions and remove the butyrate that was slowing the digestive process. Other classical microbiologists have been troubled by the fact that PCR analyses of fruit and produce have indicated the presence of potential pathogens (e.g., *Listeria* and *Escherichia coli* 0157), while cultures of the same materials have yielded negative results. The revelation that biofilm cells do not grow when spread on the surfaces of agar plates stimulated direct examinations of tissue surfaces using FISH probes and confocal microscopy, and the mystery was solved when well-developed biofilms of these organisms were found. We submit that the analysis of any natural ecosystem is incomplete and likely to become stalled by anomalous data, if we examine only planktonic populations, but that the invocation of the biofilm concept has the potential to solve many of these mysteries.

Biofilms in Industrial Ecosystems

For several decades, the control of the very serious problem of corrosion of metals by bacteria was predicated on a planktonic model, in spite of the obvious fact that the attack on stationary metals by mobile bacterial cells seems ludicrous at a basic conceptual level. Several microbial villains were designated, amongst whom the sulfate-reducing organisms (SRB) were prominent, and surveillance or the world's pipelines was initiated using an elaborate whole bottle system to detect SRBs using a lactate medium with iron filings. Sick pipelines were detected and treated with biocides to kill the deadly SRBs, but continuing metal loss caused catastrophic pipe failures, and the planktonic bacterial counts always returned when the biocide treatment was over. Biocide salesmen declared that the SRB in the most affected pipelines had become 'resistant' to the biocide in question, and offered new and better (and more expensive) biocides to save the day and keep the oil flowing. In the meantime, engineers with scant knowledge of microbiology observed that regular 'pigging' of pipelines with mechanical scrapers that removed tons of 'slime' were effective in controlling corrosion, especially when coupled with the use of biocides. David White had a medical degree, and a background in physical chemistry, and he led the team that solved the corrosion puzzle in the 1980s, by figuring out that bacterial biofilms cause corrosion by building structured communities on the metal surface that actually constitute classic 'corrosion cells'. The biofilms are a living energy-driven cathode, the metal is the matching anode, and a well-organized bacterial biofilm can drill a neat hole through 5/8 inch steel pipe in a couple of months. So the biofilm concept has solved another microbiological mystery and we now detect SRBs in biofilms (not bulk water samples), we pig all 'piggable' lines with relentless regularity, and we follow the pig with biocides to kill the bacteria we have just blasted off of the pipe wall with mechanical and shear forces. The solution to this problem is poignant, in terms of communication between scientists in the same area, because the external surfaces of the thousands of miles of metal pipe we bury in the ground are always protected from microbial corrosion by the application of 'cathodic protection'. For at least four decades, oil companies paid dearly for courses in cathodic protection, which prevents external pipe corrosion by overriding the electrical potentials of biofilm-driven corrosion cells, while sponsoring corrosion classes for the same employees, in which they were taught how to prevent internal corrosion by the use of biocides! Again, we need to know where the bacteria are, in industrial systems ranging from pipelines to cooling towers, and useful answers simply come to light when we know their locations and their mode of growth.

Biofilms in Medical Systems

The paradigm that has developed in medical microbiology is especially unfortunate, in view of the phenomenal success of the pioneers of this field in the virtual eradication of acute epidemic disease. The paradigm depends on culture methods for the detection and recovery of the bacteria that cause a particular disease, the cultivation of these bacteria in single-species cultures in defined media, and the extrapolation from culture data to define the etiology of the disease and to suggest therapeutic strategies. The success of this paradigm, in Koch's heyday and in the first part of the twentieth century, may have blinded us to its scientific faults and to the fact that it has not been successful in the definition or the treatment of the burgeoning number of chronic bacterial diseases that currently beset us. In terms of the biofilm concept, the failure of the traditional paradigm to accommodate the fact that 80% of the bacteria in modern chronic infections live in biofilms, and fail to produce colonies when spread on agar surfaces, is a pivotal deficit. But it is equally disturbing to note that any given strain of bacteria that is recovered from an infection is only one of many strains that may constitute the super genome of the species, and that any chosen 'type' strain may lack hundreds or even thousands of genes that are present in other strains in the same infection. Tragically, because of the general conservation of 'housekeeping' genes, the genes that are missing from any type strain may control important pathogenic processes, and even this depleted complement of genes may be further depleted by genetic drift as the strain is carried in serial culture. Coupled with the fact that the biofilm phenotype differs so profoundly from the planktonic phenotype of the same strain, any attempts to extrapolate from a single strain grown as planktonic cells in a defined medium to a disease caused by multiple strains growing as biofilms in infected tissues seems futile, and possibly fraudulent.

The persistence of the traditional planktonic paradigm has puzzled clinicians, many of whom see their patients suffering from infections that produce obvious symptoms and demonstrable tissue damage, while lab tests yield negative cultures. The problem of overt culture-negative infections has bedeviled clinicians in orthopedic surgery, ear nose and throat (ENT), and urology, and modern detection methods have uniformly detected bacteria where cultures have failed. Clinicians from these different specialties do not often read each other's literature, so many are unaware that the problem of culture-negative chronic infections has been solved and that large numbers of biofilm bacteria have been found in each case. At the Mayo Clinic, Robin Patel has pioneered the use of PRC-based nucleic acid technologies to detect the presence of bacteria in infections of orthopedic devices, and many new initiatives will gradually replace cultures with DNA-based methods. Roger Lasken, of the Craig Venter Institute, will soon sequence the entire genomes of a large number of single bacterial cells, isolated from a cystic fibrosis lung by micromanipulation, and we will finally know how many different strains of how many different species are present in a well-defined biofilm infection. On a practical note, it should be noted that many clinicians have ignored negative culture data, because their patients are obviously infected, and have developed empirical therapeutic strategies based on the physical removal of biofilms (where possible) and high-dose antibiotic therapy. As in natural and industrial ecosystems, horse sense has triumphed, and the modern DNA-based methods and direct observations that have brought microbial ecology forward have provided the rationale for this sensible approach.

One area in which the traditional paradigm still lingers, with very invidious effect, is in the treatment of multispecies infections. If a bacterium has been regularly cultured from a type of chronic bacterial infection, that organism is enshrined as the causative agent of that particular infection, and all strategies from prevention to therapy are predicated on that assumption. In fact, when a predisposing condition such as a burn offers a hospitable environment for members of a very large spectrum of bacterial species, many species may colonize the tissue concerned, because many organisms are present and nothing predisposes to favor colonization by any particular organism. Culture media have been devised and refined to favor the growth of certain infamous pathogens, and we can always culture *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterobacter faecalis* from wounds, the cystic fibrosis lung, and failed root canals, even though we may not be able to culture many of the organisms that coinfect with them. This preoccupation with 'pathogens of note' harkens back to the era of the 'one pathogen = one disease' theorem, and it is a virtual ecological impossibility that specific pathogens occur exclusively in lesions that are open to colonization from the environment, which is an exuberant microbiological zoo. Furthermore, there was some justification in this thesis if the pathogen possessed specific aggressive toxins and other pathogenic factors, but the common link between biofilm pathogens that cause chronic infections is that their pathology is mediated by the inflammatory response to their continued presence in the affected tissues. There is, therefore, absolutely no valid reason to suspect that a multispecies infection, like that of a wound, is caused by a single bacterial species because only one species is recovered in cultures. DNA-based analysis has revealed the presence of dozens of bacterial species in wounds that have yielded positive cultures for only *S. aureus*, and our ongoing work with FISH probes indicates that several bacterial species form biofilms in the wound bed. A detailed examination of the bacterial biofilms in the wound bed, and an analysis of the extent to which each species causes cytokine production and leukocyte mobilization, will tell us which bacterial species are involved and how they contribute to the sustained infection.

Postlude

Direct observations indicate, unequivocally, that bacteria in all ecosystems live predominantly in matrix-enclosed biofilms attached to surfaces. These sessile biofilms have many attributes that have not been previously associated with prokaryotic organisms, and it is now clear that they function as metabolically integrated communities whose sophistication and internal communications rival those of multicellular eukaryotes. As we approach conceptual problems, in all subdivisions of microbiology, the simple addition of the biofilm concept to the traditional culture-based microbiological paradigm provides us with an intellectual basis for understanding these puzzles by locating and enumerating the bacteria, of all species, that are present and active in the ecosystem.

Summary

Direct observations of bacteria growing in natural and pathogenic ecosystems have shown that these organisms grow predominantly in matrix-enclosed biofilms. These sessile bacteria assume a distinct phenotype that differs from that of their planktonic counterparts, renders them resistant to antimicrobial agents, and makes them incapable of producing colonies when dispersed on agar plates. In mature biofilms very effective metabolic interaction is achieved by the juxtaposition of cooperative species, and equally effective communication is achieved by means of electrically conductive nanowires, and by cell–cell signals that spread by simple diffusion or by transport in specialized vesicles.

Biofilms recycle organic matter with remarkable efficiency, they cooperate with many plants (e.g., legumes) and animals (e.g., ruminants), and they may exert protective effects on many tissues that they colonize to the exclusion of pathogenic species. Biofilms also mediate the attack of bacteria on metals (e.g., pipelines), by concentrating ions and electrical fields at a specific location of the affected surface, and cause damaging inflammations of tissues by allowing bacteria to persist for decades in human tissues. If we are to enhance these beneficial activities, and to thwart these destructive tendencies of biofilm bacteria, we must apply the biofilm paradigm throughout modern microbiology.

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Biological Warfare

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Glossary

biological warfare (BW) The use of microorganisms, such as bacteria, fungi, viruses, and rickettsiae, to produce death or disease in humans, animals, or plants. The use of toxins to produce death or disease is often included under the heading of BWR (US Army definition: included in Army report to the Senate Committee on Human Resources, 1977).

biological weapon(s) Living organisms, irrespective of their nature, which are intended to cause disease or death in man, animals or plants, and which depend for their effects on their ability to multiply in the person, animal, or plant attached (United Nations definition: included in the Report of the Secretary General entitled Chemical and Bacteriological (biological) Weapons and the Effects of their Possible Use, 1969).

bioterrorism The use of biological agents, such as pathogenic microorganisms or agricultural pests, to cause harm for terrorist purposes.

bioterror pathogen A microorganism used for terrorist purposes to cause disease or death in man, animals, or plants.

genetic engineering Methods by which the genomes of plants, animals, and microorganisms are manipulated, and includes but is not limited to recombinant DNA technology.

recombinant DNA technology Techniques where by different pieces of DNA are spliced together and inserted into vectors such a bacteria or yeast.

toxin weapon(s) (TW) Any poisonous substance, irrespective of its origin or method of production, which can be produced by a living organism, or any poisonous isomer, homolog, or derivative of such a substance (US Arms Control and Disarmament Agency (ACDA) definition: proposed on 20 August 1980).

weapon of mass destruction (WMD) ‘Chemical, biological, radiological, and nuclear agents (CBRN), in the possession of hostile states or terrorists’, that could ‘potentially cause mass casualties’. (Homeland Security Presidential Directive/HSPD-18, Medical Countermeasures against WMD, 31 January 2007.)

Abbreviations

ACDA	US Arms Control and Disarmament Agency
BARDA	Biomedical Advanced Research and Development Authority
BDRP	Biological Defense Research Program
BW	biological warfare
CBRN	Chemical, biological, radiological, and nuclear agents
CDC	Centers for Disease Control and Prevention
DARPA	Defense Advanced Research Project Agency
DoD	Department of Defense
NATO	North Atlantic Treaty Organization
TW	toxin weapon
USAMRIID	US Army Medical Research Institute of Infectious Diseases
WMD	weapon of mass destruction

Defining Statement

The most general concept of biological warfare (BW) involves the use of any biological agent as a weapon directed against humans, animals, or crops with the intent to kill, injure, or create a sense of havoc against a target population. This agent could be in the form of a viable organism or a metabolic product of the organism, such as a toxin. This article will focus on the use of viable biological agents because many of the concepts relating to the use of toxins are more associated with chemical warfare. The use of viable organisms or viruses involves complex issues that relate to containment. Once such agents are released, even in relatively small numbers, the area of release has the potential to enlarge to a wider population due to the ability of the viable agent to proliferate while spreading from one susceptible host to another.

Introduction

During the last decade of the twentieth century and early years of the twenty-first century, several events marked significant alterations in the concept of biological warfare (BW). These events include the end of the Cold War, the open threat by Iraq of using BW agents in the First Gulf War, the events of 9/11 followed by dissemination of anthrax through the US Mail system, and the war with Iraq over possible weapon of mass destruction (WMD). These events lead to the full realization that, in addition to BW between nations, the developed world is quite susceptible to attack by radical terrorists employing BW agents. This was a major expansion in the concept of BW and transformed the subject, once limited to the realm of political and military policy makers, to a subject that must be considered by a wide range of urban disaster planners, public health officials, and the general public. BW is a complex subject that is difficult to understand without a basic knowledge of a long and convoluted history. BW can be traced to ancient times and have evolved into more sophisticated forms with the maturation of the science of bacteriology and microbiology. It is important to understand the history of the subject because one often has preconceived notions of BW that are not based on facts or involve concepts more related to chemical rather than biological warfare. Many of the contemporary issues relating to BW deal with third-world conflicts, terrorist groups, or nonconventional warfare. An understanding of these issues becomes important because many of the long-standing international treaties and conventions on BW were formulated in an atmosphere of either international conflict or during the Cold War period of international relations. Many of the classic issues have undergone significant alteration by more recent events. The issue of BW is intimately bound to such concepts as offensive versus defensive research or to the need for secrecy and national security. It is obvious that BW will continue to be a subject that will demand the attention of contemporary and future students of microbiology as well as a wide range of policy and scientific specialists.

Historical Review

300 to 1925 BC

Many early civilizations employed a crude method of warfare that could be considered BW as early as 300 BC, when the Greeks polluted the wells and drinking water supplies of their enemies with the corpses of animals. Later the Romans and Persians used the same tactics. All armies and centers of civilization need palatable water to function, and it is clear that well pollution was an effective and calculated method for gaining advantage in warfare. In 1155, at a battle in Tortona, Italy, Barbarossa broadened the scope of BW, using the bodies of dead soldiers and animals to pollute wells. Evidence indicates that well poisoning was a common tactic throughout the classical, medieval, and Renaissance periods. In more modern times, this method has been employed as late as 1863 during the US Civil War by General Johnson, who used the bodies of sheep and pigs to pollute drinking water at Vicksburg.

The wide use of catapults and siege machines in medieval warfare introduced a new technology for delivering biological entities. In 1422 at the siege of Carolstein, catapults were used to project diseased bodies over walled fortifications, creating fear and confusion among the people under siege. The use of catapults as weapons was well established by the medieval period, and projecting diseased bodies over walls was an effective strategy employed by besieging armies. The siege of a well-fortified position could last for months or years, and it was necessary for those outside the walls to use whatever means available to cause disease and chaos within the fortification. This technique became commonplace, and numerous classical tapestries and works of art depict diseased bodies or the heads of captured soldiers being catapulted over fortified structures.

In 1763, the history of BW took a significant turn from the crude use of diseased corpses to the introduction of a specific disease, smallpox, as a weapon in the North American Indian wars. It was common knowledge at the time that the Native American population was particularly susceptible to smallpox, and the disease may have been used as a weapon in earlier conflicts between European settlers and Native Americans. In the spring of 1763, Sir Jeffrey Amherst, the British Commander-in-Chief in North America, believed the western frontier, which ran from Pennsylvania to Detroit, was secure, but the situation deteriorated rapidly over the next several months. The Indians in western Pennsylvania were becoming particularly aggressive in the area around Fort Pitt, near what is now Pittsburgh. It became apparent that unless the situation was resolved, western Pennsylvania would be deserted and Fort Pitt isolated. On 23 June 1763, Colonel Henry Bouquet, the ranking officer for the Pennsylvania frontier, wrote to Amherst, describing the difficulties Captain Ecuyer was having holding the besieged Fort Pitt. These difficulties included an outbreak of smallpox among Ecuyer's troops. In his reply to Bouquet, Amherst suggested that smallpox be sent among the Indians to reduce their numbers. This well-documented suggestion is significant because it clearly implies the intentional use of smallpox as a weapon. Bouquet responded to Amherst's suggestion stating that he would use blankets to spread the disease.

Evidence indicates that Amherst and Bouquet were not alone in their plan to use BW against the Indians. While they were deciding on a plan of action, Captain Ecuyer reported in his journal that he had given two blankets and a handkerchief from the garrison smallpox hospital to hostile chiefs with the hope that it would spread the disease. It appears that Ecuyer was acting on his own and did not need persuasion to use whatever means necessary to preserve the Pennsylvania frontier. Evidence also shows that the French used smallpox as a weapon in their conflicts with the native population.

Smallpox also played a role in the American Revolutionary War, but the tactics were defensive rather than offensive: British troops were inoculated against smallpox, but the rebelling American colonists were not. This protection from disease gave the British an advantage for several years, until Washington ordered inoculation against smallpox for all American troops.

It is clear that by the eighteenth century BW had become disease-oriented, even though the causative agents and mechanisms for preventing the spread of diseases were largely unknown. The development of the science of bacteriology in the nineteenth and early twentieth centuries considerably expanded the scope of potential BW agents. In 1915, Germany was accused of using cholera in Italy and plague in St. Petersburg. Evidence shows that Germany used glanders and anthrax to infect horses and cattle, respectively, in Bucharest in 1916 and employed similar tactics to infect 4500 mules in Mesopotamia the following year. Germany issued official denials of these accusations. Although there apparently was no large-scale battlefield use of BW in World War I, numerous allegations of German use of BW were made in the years following the war. Britain accused Germany of dropping plague bombs, and the French claimed the Germans had dropped disease-laden toys and candy in Romania. Germany denied the accusations.

Although chemical warfare was far more important than BW in World War I, the general awareness of the potential of biological weapons led the delegates to the Geneva Convention to include BW agents in the 1925 Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare. The significance of the treaty will be discussed later (see 'International treaties').

1925–90

The tense political atmosphere of the period following the 1925 Geneva Protocol and the lack of provisions to deter biological weapons research had the effect of undermining the treaty. The Soviet Union opened a BW research facility north of the Caspian Sea in 1929; the United Kingdom and Japan initiated BW research programs in 1934. The Japanese program was particularly ambitious and included experiments on human subjects prior to and during World War II.

Two factors were significant in mobilizing governments to initiate BW research programs: (1) a continuing flow of accusations regarding BW and (2) the commitment of resources for BW research by several national adversaries, thus creating a feeling of insecurity among governments. The presence of BW research laboratories in nations that were traditional or potential adversaries reinforced this insecurity. Thus, despite the Geneva Protocol, it was believed that it was politically unwise for governments to ignore the threat of BW, and the result was the use of increasingly sophisticated biological weapons.

In 1941, the United States and Canada joined other nations and formed national programs of BW research and development. Camp Detrick (now Fort Detrick) became operational as the center for US BW research in 1943, and in 1947 President Truman withdrew the Geneva Protocol from Senate consideration, citing current issues such as the lack of verification mechanisms that invalidated the underlying principles of the treaty. However, there was no widespread use of BW in a battlefield setting during World War II. BW research, however, continued at an intense pace during and after the war. By the end of the decade, the United States, the United Kingdom, and Canada were conducting collaborative experiments involving the release of microorganisms from ships in the Caribbean. In 1950, the US Navy conducted open-air experiments in Norfolk, Virginia, and the US Army conducted a series of airborne microbial dispersals over San Francisco using *Bacillus globigii*, *Serratia marcescens*, and inert particles.

Not surprisingly, the intense pace of BW research led to new accusations of BW use, most notably by China and North Korea against the United States during the Korean War. In 1956, the United States changed its policy of 'defensive use only' to include possible deployment of biological weapons in situations other than retaliation. During the 1960s, all branches of the US military had active BW research programs, and additional open-air dissemination experiments with stimulants were conducted in the New York City subway system. By 1969, however, the US military concluded that BW had little tactical value in battlefield situations, and since it was felt that in an age of nuclear weapons dominated the strategic equation, the United States would be unlikely to need or use BW. Thus, President Nixon announced that the United States would unilaterally renounce BW and eliminate stockpiles of biological weapons. This decision marked a turning point in the history of BW: Once the US government made it clear that it did not consider biological weapons a critical weapon system, the door was opened for negotiation of a strong international treaty against BW.

Once military strategists had discounted the value of BW, an attitude of openness and compromise on BW issues took hold, leading to the 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction (see 'International treaties'). The parties to the 1972 Convention agreed to destroy or convert to peaceful use all organisms, toxins, equipment, and delivery systems. Following the signing of the 1972 treaty, the US government generated much publicity about its compliance activities, inviting journalists to witness destruction of biological weapons stockpiles.

The problem of treaty verification, however, beleaguered the 1972 Convention. Press reports accusing the Soviet Union of violating the treaty appeared as early as 1975. When an outbreak of anthrax was reported in Sverdlovsk, Soviet Union, in 1979, the United States claimed it was caused by an incident at a nearby Soviet biological defense laboratory that had released anthrax spores into the surrounding community. The Soviet government denied this allegation, claiming the outbreak was caused by contaminated black market meat.

BW continued to be discussed in the public media throughout the 1980s. In 1981, reports describing the American 'cover-up' of Japanese BW experiments on prisoners of war began to surface in the public and scientific literature. In 1982, *The Wall Street Journal* published a series of articles on Soviet genetic engineering programs that raised many questions about the scope of Soviet BW activities. The environmental effects of testing biological agents at Dugway Proving grounds in Utah received considerable press attention in 1988, leading to a debate over the need for such a facility.

The 1980s also were characterized by debate over larger issues relating to BW. A public debate in 1986 considered the possible role of biological weapons in terrorism. Scientific and professional societies, which had avoided the issues for many years, began considering both specific issues, such as Department of Defense (DoD) support for biological research and more general issues, such as adopting ethical codes or guidelines for their members.

1990 and Contemporary Developments

The last decade of the twentieth century and the early years of the twenty-first century saw a remarkable transition in the concepts relating to BW. With the fall of the Soviet Union and the rise of the United States as the only remaining superpower, many policies that were in place in a dual superpower setting became altered. Although the concept of BW as an alternative offensive weapon among developing and nonnuclear States remained a serious concern, the new emphasis was on the threat of these weapons being used by bioterrorist groups. The events of 11 September 2001, followed by a US anthrax scare, dramatically shifted awareness to the need for improved Public Health protection for the general population, the initiation, and placement of early and rapid detection systems for potential biological agents and increased border protection. The concept of BW agents in the hands of terrorists was not new, however, and the foundation for shift in emphasis from nations to independent terrorist groups had begun in the 1990s.

Prior to the 1990s, most US defensive research and BW-related policies were directed to counter potential BW use by the Soviet Union. As the wall of Soviet secrecy eroded during the 1990s, the extent of the Soviet BW program became apparent. There was international concern that a large number of unemployed BW researchers could find work as advisors for developing countries that viewed BW as a rational defense strategy, especially those countries without nuclear capability or those without restrictive laws against radical terrorist groups. Also, the open threat by the Iraqi military to use BW agents raised serious concerns and changed attitudes about BW. The plans for Operation Desert Storm included provisions for protective equipment and prophylactic administration of antibiotics or vaccines to protect against potential biological weapons. Many of the critics of the US Biological Defense Research Program (BDRP) were now asking why the country was not better prepared to protect its troops against biological attack. BW was not used during the First Gulf War, but the threat of its use provided several significant lessons. Although there was considerable concern that genetic engineering would produce new, specialized biological weapons, most experts predicted that 'classical' BW agents, such as anthrax and botulism, would pose the most serious threats to combat troops in Operation Desert Storm. Efforts by the United Nations after the war to initiate inspection programs demonstrated the difficulty of verifying the presence of production facilities for BW agents; these difficulties highlight the need for verification protocols for the BW Convention. Verification and treaty compliance are prominent contemporary BW issues. Following the Gulf War, the actual extent of the intense Iraqi BW research programs was understood based on information from defectors. The actual programs were huge compared to predicted estimates of the Iraqi BW program as well as the postwar 'verification' programs that essentially uncovered very little. This vast discrepancy demonstrated the inadequacy verification procedures.

The World Trade Center attack and the anthrax incidents in 2001 were the most significant contemporary developments contributing to the realization that urban centers, public facilities, and the general population are all vulnerable to attack by terrorists employing BW agents. Local and national governments realized the extent of the vulnerability and began taking extensive measures to formulate policies to address potential bioterror attacks. In the United States, it was felt that the various agencies involved in protecting the population against the threat of bioterrorism had to be strengthened. The US Patriot Act of October 2001 and the formation of the Department of Homeland Security are just some of the steps taken to address the needs of responding to and limiting the threat of a BW or bioterrorist attack in the US. Canada and several European countries have created new or greatly enhanced existing government agencies, such as the European Centre for Disease Control, to protect the population against bioterrorism and other major potential disasters. Much work remains to be accomplished in this area.

Additionally, in the United States, a number of legislative acts and directives that were designed to increase the nation's biodefense capabilities and responsiveness were approved. Increased resources have been made available through the Department of Health and Human Services in support of medical countermeasure preparedness. On 21 July 2004, President George W. Bush signed the Project BioShield Act of 2004 (Project Bioshield). The purpose of this bill was to accelerate the research, development, acquisition, and availability of safe and effective countermeasures against Chemical, Biological, Radioactive, and Nuclear Threats by providing the funding to purchase countermeasures. Under this bill, the HHS pursued acquisition of countermeasures for anthrax, smallpox, botulinum toxins, and radiological/nuclear agents. Project Bioshield created a $5.6 billion special reserve fund for use over 10 years (FY04-FY13) to acquire medical countermeasures. New legislation, the Pandemic and All-Hazards Prepardedness Act was approved in December 2006. This act provided for the establishment of the Biomedical Advanced Research and Development Authority (BARDA) within HHS. BARDA is intended to address the inadequacy of Project Bioshield which, while providing funds for procurement of the actual end product, did not allow for funding of the actual research and development. BARDA should have addressed the so-called valley of death where there is no funding available to support the expensive later stage development. BARDA is intended to provide direct funding of medical countermeasure advanced research and development (Federal Register/Vol. 72, No. 53/Tuesday, 20 March 2007, p. 13109).

International Treaties

The 1925 Geneva Protocol

The 1925 Geneva Protocol was the first international treaty to place restrictions on BW. The Geneva Protocol followed a series of international agreements that were designed to prohibit the use in war of weapons that inflict or prolong unnecessary suffering of combatants or civilians. The St. Petersburg Declaration of 1868 and the International Declarations concerning the Laws and Customs of War, which was signed in Brussels in 1874, condemned the use of weapons that caused useless suffering. Two major international conferences were held at The Hague in 1899 and 1907. These conferences resulted in declarations regarding the humanitarian conduct of war. The conference regulations forbid nations from using poison, treacherously wounding enemies, or using munitions that would cause unnecessary suffering. The so-called Hague Conventions also prohibited the use of projectiles to diffuse asphyxiating or deleterious gases. The Hague Conventions still provide much of the definitive law of war as it exists today.

The Hague Conventions did not specifically mention BW, due in part to the lack of scientific understanding of the cause of infectious diseases at that time. The Conventions have, however, been cited as an initial source of the customary international laws that prohibit unnecessary suffering of combatants and civilians in war. While biological weapons have been defended as humanitarian weapons, on the grounds that many biological weapons are incapacitating but not lethal, there are also biological weapons that cause a slow and painful death. It can be argued, therefore, that the Hague Conventions helped to set the tone of international agreements on laws of war that led to the 1925 Geneva Protocol.

The 1925 Geneva Protocol, formally called the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare, was opened for signature on 17 June 1925 in Geneva, Switzerland. More than 100 nations have signed and ratified the protocol, including all members of the Warsaw Pact and North Atlantic Treaty Organization (NATO). The 1925 Geneva Protocol was initially designed to prevent the use of chemical weapons in war; however, the protocol was extended to include a prohibition on the use of Bacteriological Methods of Warfare. The Geneva Protocol distinguishes between parties and nonparties by explicitly stating that the terms of the treaty apply only to confrontations in which all combatants are parties and when a given situation constitutes a 'war'. Additionally, a number of nations ratified the Geneva Protocol with the reservation that they would use biological weapons in retaliation against a biological weapons attack. This resulted in the recognition of the Geneva Protocol as a 'no first use' treaty.

The 1972 BW Convention

International agreements governing BW have been strengthened by the 1972 BW Convention, which is officially called the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapon and on Their Destruction. The Convention was signed simultaneously in 1972 in Washington, London, and Moscow and entered into force in 1975. The preamble to the 1972 BW Convention states the determination of the state's parties to the treaty to progress toward general and complete disarmament, including the prohibition and elimination of all types of WMD. This statement places the Convention in the wider setting of international goals of complete disarmament. The 1972 BW Convention is also seen as a first step toward chemical weapons disarmament.

The 1972 BW Convention explicitly builds on the Geneva Protocol by reaffirming the prohibition of the use of BW in war. The preamble, although not legally binding, asserts that the goal of the Convention is to completely exclude the possibility of biological agents and toxins being used as weapons and states that such use would be repugnant to the conscience of humankind. The authors of the 1972 Convention, therefore, invoked societal attitudes as justification for the existence of the treaty.

In 1972 BW Convention evolved, in part, from a process of constant reevaluation of the Geneva Protocol. From 1954 to the present, the United Nations has periodically considered the prohibition of chemical and biological weapons. The Eighteen-Nation Conference of the Committee on Disarmament, which in 1978 became the Forty-Nation Committee on Disarmament, began talks in 1968 to ban chemical weapons. At this time, chemical, toxin, and biological weapons were being considered together, in an attempt to develop a comprehensive disarmament agreement. However, difficulties in reaching agreements on chemical warfare led to a series of separate negotiations that covered only BW and TWs. The negotiations resulted in the drafting of the 1972 BW Convention.

The 1972 BW Convention consists of a preamble, followed by 15 articles. Article I forms the basic treaty obligation. Parties agree never, under any circumstances, to develop, produce, stockpile, or otherwise acquire or retain the following:

1. Microbial or other biological agents, or toxins irrespective of their origin or method of production, of types and in quantities that have no justification for prophylactic, protective, or other peaceful purposes.
2. Weapons, equipment, or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict.

Article II requires each party to destroy, or divert to peaceful purposes, all agents, toxins, equipment, and delivery systems that are prohibited in Article I and are under the jurisdiction or control of the party. It also forbids nations from transferring, directly or indirectly, materials specified in Article I and prohibits nations from encouraging, assisting, or inducing any state, group of states, or international organizations from manufacturing or acquiring the material listed in Article I. There is no specific mention of subnational groups, such as terrorist organizations, in the treaty.

Article IV requires each party to the Convention to take any measures to ensure compliance with the terms of the treaty. Article IV has been interpreted by some states as the formulation of civil legislation or regulations to assure adherence to the Convention. This civil legislation could regulate activities by individuals, government agencies, universities, or corporate groups.

Articles V–VII specify procedures for pursuing allegations of noncompliance with the 1972 BW Convention. The United Nations plays an integral part in all of the procedures for investigating allegations of noncompliance. According to Article VI, parties may lodge a complaint with the Security Council of the United Nations if a breach of the treaty is suspected. All parties must cooperate with investigations that may be initiated by the Security Council. Article VII requires all parties to provide assistance or support to any party that the Security Council determines has been exposed to danger as a result of violation of the Convention. Articles VII–IX are general statements for obligations of the parties signing the protocol. Article X gives the parties the right to participate in the fullest possible exchange of equipment, materials, and scientific or technological information of the use of bacteriological (biological) agents and toxins for peaceful purposes. Article XI allows parties to propose amendments to the Convention. The amendments only apply to those states that accept them and enter into force after a majority of the states parties to the Convention have agreed to accept and be governed by the amendment.

Article XII requires that a conference be held 5 years after the entry into force of the BW Convention. Article XIV states that the 1972 BW Convention is of unlimited duration. A state party to the treaty is given the right to withdraw from the treaty if it decides that extraordinary events, related to the subject matter of the Convention, have jeopardized the supreme interests of the country. This article also opens the Convention to all nations for signature. Nations that did not sign the Conventions before its entry into force may accede to it at any time.

Review Conferences

The 1972 Convention contained a stipulation that a conference be held in Geneva 5 years after the terms of the Convention entered into force. The purpose of the conference was to review the operation of the Convention and to assure that the purposes of the Convention were being realized. The review was to take into account any new scientific and technological developments that were relevant to the Convention. The first review conference was held in Geneva in 1980. Several points contained in the original Convention were clarified at this conference. There was general agreement that these conferences would serve a definite function in solving contemporary problems, such as issues of verification and compliance that need clarification based on changing events. While limited in scope, these conferences made some progress in keeping the 1972 Convention relevant to the needs of a changing world situation.

Select US Laws and Acts

A number of US laws have been enacted since 1989 that impact on BW:

Biological Weapons and Anti-Terrorist Act (1989): Established as a federal crime, the development, manufacture, transfer, or possession of any biological agent, toxin, or delivery system for use as a weapon.

Chemical and Biological Weapons Control Act (1991): Places sanctions on companies that knowingly export goods or technologies relating to biological weapons to designated prohibited nations.

The Defense Against WMD Act (1996): Designed to enhance federal, state, and local emergency response capabilities to deal with terrorist incidents.

Antiterrorism and Effective Death Penalty Act (1996): Established as a criminal act, any threat or attempt to develop BW or DNA technology to create new pathogens or make more virulent forms of existing organisms.

National Laboratory Response Network (1999): A joint effort by the Centers for Disease Control and Prevention (CDC) and US Army Medical Research Institute of Infectious Diseases (USAMRIID) to establish a network of public health laboratories throughout the United States, and was reinforced and expanded following the anthrax letter incident of 2001.

The USA Patriot Act (2001): Greatly expanded the ability of law enforcement agencies for fighting terrorism in the United States and abroad. New crime categories, such as domestic terrorism, were established. Under this act laws on immigration, banking, money laundering, and foreign intelligence have been amended.

Public Health Security and Bioterrorism Preparedness and Response Act of 2002: Encouraged technological solutions to prevent the threat of bioterrorism and the stockpiling of vaccines and supplies.

Homeland Security Act (2003): Attempt to consolidate 25 agencies and tens of thousands government employees into a new department to prevent terrorist attacks, reduce vulnerability, and minimize damage and recover from attacks that may occur.

Project BioShield Act of 2004 (Project Bioshield): Provides funds to purchase countermeasures against Chemical, Biological, Radioactive, and Nuclear Threats.

Pandemic and All-Hazards Prepardedness Act of 2006: Included provisions for the establishment of the BARDA within HHS. Funding provided through BARDA will support medical countermeasure advanced research and development.

CDC 42 CFR Part 1003 Possession, use, and Transfer of Select Agents and Toxins: Established a rule regarding possession, use, and transfer of select agents and toxins that pose a significant risk to public health and safety. Infectious agents have been added to this list at several intervals over time. Some of the organisms regulated by CDC are listed in **Table 1**.

Table 1 Representative organisms regulated by CDC (Center for Disease Control)

Bacteria	*Viruses*
Bacillus anthracis	Arenaviruses
Brucella species	Crimean-Congo hemorrhagic fever virus
Burkholderia mallei, *B. pseudomallei*	Eastern equine encephalitis virus
Clostridium botulinum, *B. perfringens*	Ebola virus
Escherichia coli 0157:H7	Equine morbillivirus
Francisella tularensis	Hantavirus
Salmonella species	Lassa fever virus
Shigella species	Marburg virus
Vibrio cholerae	Monkeypox virus
	Nipah virus
Yersinia pestis	Rift Valley fever virus
	South American hemorrhagic fever viruses
Coccidioides immitis	Tick-borne encephalitis complex viruses
Rickettsiae and Chlamydia	Variola (smallpox) major virus
Chlamydia psittaci	Venezuelan equine encephalitis virus
Coxiella burnetii	Western equine encephalitis virus
Rickettsia prowazekii	Yellow fever virus
Rickettsia rickettsii	
Select Genetic Elements, Recombinant Nucleic Acids, and Recombinant Organisms	

Current Research Programs

The US BDRP today is headquartered at the USAMRIID at Fort Detrick, Maryland. USAMRIID is an organization of the US Army Medical Research and Materiel Command. In accordance with official US policy, the BDRP is solely defensive in nature, with the goal of providing methods of detection for, and protective countermeasures against, biological agents that could be used as weapons against US forces or civilians by hostile states or individuals. USAMRIID plays a key role as the only laboratory in the US DoD equipped for the safe study of highly hazardous infectious agents that require maximum containment at biosafety level (BSL)-4.

Current US policy stems from the 1969 declaration made by President Nixon that confined the US BW program to research on biological defense such as immunization and measures of controlling and preventing the spread of disease. Henry Kissinger further clarified the US BW policy in 1970 by stating that the US biological program will be confined to research and development for defensive purposes only. This did not preclude research into those offensive aspects of biological agents necessary to determine what defensive measures were required. The BDRP expanded significantly in the 1980s, in an apparent response to alleged treaty violations and perceived offensive BW capabilities in the Soviet Union. These perceptions were espoused primarily by representatives of the Reagan Administration and the Department of State. At congressional hearings in May 1988, the US government reported that at least ten nations, including the Soviet Union, Libya, Iran, Cuba, Southern Yemen, Syria, and North Korea, were developing biological weapons. Critics of the US program refuted the need for program expansion.

The BDRP is focused in three sites, the USAMRIID at Fort Detrick, Maryland; Aberdeen Proving Ground in Maryland; and the Dugway Proving Ground in Utah. USAMRIID is designated as the lead laboratory in medical defense against BW threats. Research conducted at the USAMRIID focuses on medical defense such as the development of vaccines and treatments for both natural diseases and potential BW agents. Work on the rapid detection of microorganisms and the diagnosis of infectious diseases are also conducted. The primary mission at the Aberdeen Proving Ground is nonmedical defense against BW threats including detection research, such as the development of sensors and chemiluminescent instruments to detect and identify bacteria and viruses, and development of methods for material and equipment decontamination. The US Army Dugway Proving Ground is a DoD major range and test facility responsible for development, test, evaluation, and operation of chemical warfare equipment, obscurants and smoke munitions, and biological defense equipment. Its principal mission with respect to the BDRP is to perform developmental and operational testing for biological defense material, including the development and testing of sensors, equipment, and clothing needed for defense against a BW attack.

The BDRP focuses on five main areas:

1. Development of vaccines.
2. Development of protective clothing and decontamination methods.
3. Analysis of the mode of action of toxins and the development of antidotes.
4. Development of broad-spectrum antiviral drugs for detecting and diagnosing BW agents and toxins.
5. Utilization of genetic engineering methods to study and prepare defenses against BW and toxins.

The BDRP has often been a center of controversy in the United States. One BDRP facility, the Dugway Proving Ground, was the target of a lawsuit that resulted in the preparation of the environmental impact statement for the facility. A proposal for a high-level containment laboratory (designated P-4) was ultimately changed to new plans for a lower-level (P-3) facility.

The use of genetic engineering techniques in BDRP facilities has also been a focus of controversy. The BDRP position is that genetic engineering will be utilized if deemed necessary. The DoD stated that testing of aerosols of pathogens derived from recombinant DNA methodology is not precluded if a need should arise in the interest of national defense.

Many secondary sites have received and continued to obtain contracts for biological defense research. Once specific program requires a special note. DARPA (Defense Advanced Research Project Agency) is a Pentagon program that invests significantly in pathogen research through grants to qualified institutions. This project initially focused on engineering and electronics (computer) projects; however, starting in 1995 biology became a key focus, and several BW-defensive research grants are now in operation at many academic and private institutions.

An important issue in biological defense has been the convergence in the late 1990s and the early twenty-first century of the increased need for new biological countermeasures at the same time that private pharmaceutical company research and development in infectious diseases has diminished. Numerous factors have been outlined by a variety of organizations, including the Infectious Disease Society of America, that provide reasons for the decreasing activity of the private sector in infectious diseases research and development. A major component of this is the low return on investment of infectious disease pharmaceutical agents compared to agents in other therapy areas such as oncology and diabetes. The concern about diminished research and development in infectious diseases has been met with a number of initiatives by the US government and other groups to try to stimulate companies to invest in infectious disease countermeasures.

The two major programs in the United States have been (1) the 2004 Project Bioshield and (2) the 2006 Pandemic and All-Hazards Prepardedness Act that provided for the establishment of the BARDA within HHS. These actions have the goal of obtaining medical countermeasures for both potential bioterror agents and also naturally occurring infectious disease outbreaks, either by providing funds for purchasing the countermeasures or by providing financial support for research and development activities (see '1990 and Contemporary Developments').

Very little is written in the unclassified literature on BW research conducted in countries other than the United States. Great Britain has maintained the Microbiological Research Establishment at Porton Down; however, military research is highly classified in Great Britain and details regarding the research conducted at Porton are unavailable.

During the 1970s and 1980s, a great deal of US BW policy was based on the assumption of Soviet offensive BW capabilities. Most US accounts of Soviet BW activities were unconfirmed accusations or claims about treaty violations. The Soviet Union was a party to both the 1925 Geneva Protocol and the 1972 BW Convention. According to Pentagon sources, the Soviet Union operated at least seven top-security BW centers. These centers were reported to be under strict military control. While the former Soviet Union proclaimed that their BW program was purely defensive, the United States has consistently asserted that the Soviet Union was conducting offensive BW research.

Contemporary Issues

Genetic Engineering

There has been considerable controversy over the potential for genetically engineered organisms to serve as effective BW agents. Recombinant DNA technology has been cited as a method for creating novel, pathogenic, microorganisms. Theoretically, organisms could be developed that would possess predictable characteristics, including antibiotic resistance, altered modes of transmission, or altered pathogenic and immunogenic capabilities. This potential for genetic engineering to significantly affect the military usefulness of BW has been contested. It has been suggested that because a large number of genes must work together to endow an organism with pathogenic characteristics, the alteration of a few genes with recombinant DNA technology is unlikely to yield a novel pathogen that is significantly more effective or usable than conventional BW agents.

The question of predictability of the behavior of genetically engineered organisms was addressed at an American Society for Microbiology symposium held in June 1985. Some symposium participants believed that the use of recombinant DNAs increases predictability because the genetic change can be precisely characterized. Other participants, however, felt that the use of recombinant DNA decreases predictability, because it widens the potential range of DNA sources. Other evidence supports the view that genetically engineered organisms do not offer substantial military advantage over conventional BW. Studies have shown that genetically engineered organisms do not survive well in the environment. This fact has been cited as evidence that these organisms would not make effective BW agents.

Despite the contentions that genetic engineering does not enhance the military usefulness of BW, a significant number of arguments support the contrary. At the 1986 Review Conference of the BW Convention, it was noted that genetic engineering advances since the Convention entered into force may have made biological weapons a more attractive military option.

Several authors have contended that the question of the potential of genetic engineering to enhance the military usefulness of BW is rhetorical, because the 1972 BW Convention prohibits development of such organisms despite their origin or method of production. Nations participating in both the 1980 and 1986 review conferences of the BW Convention accepted the view that the treaty prohibitions apply to genetically engineered BW agents. An amendment to the treaty, specifically mentioning genetically engineered organisms, was deemed to be unnecessary. Additionally, the United States, Great Britain, and the Soviet Union concluded in a 1980 briefing paper that the 1972 BW Convention fully covered all BW agents that could result from genetic manipulation.

While the utility of genetic engineering for enhancing the military usefulness of BW agents has been questioned, the role of genetic engineering for strengthening defensive measures against BW has been clear. Genetic engineering has the potential for improving defenses against BW in two ways: (1) vaccine production and (2) sensitive identification and detection systems. The issues of the new technologies in defensive research have been evident in the US BW program. Since 1982, US Army scientists have used genetic engineering to study and prepare defenses against BW agents. Military research utilizing recombinant DNA and hybridoma technology include the development of vaccines against a variety of bacteria and viruses, methods of rapid detection and identification of BW agents, and basic research on protein structure and gene control. By improving defenses against BW, it is possible that genetic engineering may potentially reduce the risk of using BW.

The primary effect of BW on the government regulations on genetic engineering is the tendency toward more stringent control of the technologies. The fear of genetically engineered BW agents has prompted proposals for government regulation of BW research utilizing genetic engineering research. The DoD has released a statement indicting that all government research was in compliance with the 1972 BW Convention. The government has also prepared an environmental impact statement of research conducted at Fort Detrick.

Government regulations on genetic engineering also affect BW research through limitations on exports of biotechnology information, research products, and equipment. In addition to controls of exports due to competitive concerns of biotechnology companies, a substantial amount of information and equipment related to genetic engineering is prohibited from being exported outside the United States. The Commerce Department maintains a ‘militarily critical technology’ list, which serves as an overall guide to restricted exports. Included on the list are containment and decontamination equipment for large production facilities, high-capacity biological reactors, separators, extractors, dryers, and nozzles capable of disseminating biological agents in a fine mist.

Genetic engineering has altered the concept of BW. A current, comprehensive discussion of BW would include both naturally occurring and potential genetically engineered agents. Many current defenses against BW are developed with genetic engineering techniques. Government regulations on biotechnology have limited BW research, while fears of virulent genetically engineered BW agents have strengthened public support for stronger regulations. Future policies related to BW will need to be addressed in light of their altered status.

Mathematical Epidemiology Models

While genetic engineering may potentially alter characteristics of BW agents, mathematical models of epidemiology may provide military planners with techniques for predicting the spread of a released BW agent. One of the hindrances that has prevented BW from being utilized or even seriously considered by military leaders has been the inability to predict the spread of a BW agent once it has been released into the environment. Without the capability to predict the spread of the released organisms, military planners would risk the accidental exposure of their own troops and civilians to their own weapons. The development of advanced epidemiology models may provide the necessary mechanisms for predicting the spread of organisms that would substantially decrease the deterrent factor of unpredictability.

Low-Level Conflict

Another important factor that has affected the current status of BW is the increase in low-level conflict or the spectrum of violent action below the level of small-scale conventional war, including terrorism and guerrilla warfare. In the 1980s, the low-intensity conflict doctrine, which was espoused by the Reagan administration, was a plan for US aid to anti-Communist forces throughout the world as a way of confronting the Soviet Union without using US combat troops. The significant changes in the world since the inception of the low-intensity conflict doctrine have only increased the probability of increasing numbers of small conflicts. Although no evidence indicated that the United States would consider violating the 1972 BW Convention and support biological warfare, the overall increase in low-level conflicts in the future may help create an environment conducive to the use of BW.

While BW may not be assessed as effective weapons in a full-scale conventional war, limited use of BW agents may be perceived as advantageous in a small-scale conflict. While strong deterrents exist for nuclear weapons, including unavailability and, most formidably, the threat of uncontrolled worldwide ‘nuclear winter’, BW may be perceived as less dangerous. Additionally, the participants of low-level conflicts may not possess the finances for nuclear or conventional weapons. BW agents, such as chemical weapons, are relatively inexpensive compared to other weapon systems and may be seen as an attractive alternative to the participants and leaders of low-level conflicts. Low-level conflict, therefore, increases the potential number of forums for the use of BW.

Terrorism

The most significant factor that has altered the concept of BW is the global threat of terrorism. Although there have been isolated incidents to date of the use of biological pathogens by terrorist groups, these attempts have only had minimum impact. However, the possibility of a major incident by bioterrorists is predicted as inevitable by many experts.

The relationship of terrorism and BW can be divided into two possible events. The first involves terrorist acts against laboratories conducting BW-related research. The level of security at Fort Detrick is high, the possibility of a terrorist attack has been anticipated, and contingency plans have been made. Complicating the problem of providing security against terrorist attack in the United States is the fact, that, while most BW research projects are conducted with the BW research program of the DoD, an increasing number of projects are supported by the government that are conducted outside of the military establishment. These outside laboratories could be potential targets.

The second type of terrorist event related to BW is the potential use of BW by terrorists against urban areas or major public facilities. Biological weapons are relatively inexpensive and easy to develop and produce compared to conventional, nuclear, or chemical weapons. BW agents can be concealed and easily transported across borders or within countries. Additionally, terrorists are not hampered by a fear of an uncontrolled spread of the BW agent into innocent civilian populations. To the contrary, innocent civilians are often the intended targets of terrorist activity, and the greater chance for spread of the BW is considered to be a positive characteristic for a bioterrorist. The use of these agents to attack agricultural operations or food supplies, although not directly targeting humans, can have an enormous economic impact. All aspects of BW are receiving needed attention as the potential threat of their use by terrorists is further realized by policy makers. Environmental monitoring in public places along with new technologies for rapid identification have been greatly stimulated by the threat of bioterrorism and will continue to receive much attention in the foreseeable future.

Offensive Versus Defensive BW Research

The distinctions between 'offensive' and 'defensive' BW research have been an issue since 1969, when the United States unilaterally pledged to conduct only defensive research. The stated purpose of the US BDRP is to maintain and promote national defense from BW threats. Although neither the Geneva Convention nor the 1972 Convention prohibits any type of research, the only research that nations have admitted to conducting is defensive. The problem is whether or not the two types of research can be differentiated by any observable elements.

Although production of large quantities of a virulent organism and testing of delivery systems has been cited as distinguishing characteristics of an offensive program, a substantial amount of research leading up to these activities including isolating an organism, then using animal models to determine pathogenicity, could be conducted in the name of defense.

Vaccine research is usually considered 'defensive', whereas increasing the virulence of a pathogen and producing large quantities is deemed 'offensive'. However, a critical component of a strategic plan to use biological weapons would be the production of vaccines to protect the antagonist's own personnel (unless self-annihilation was also a goal). This means that the intent of a vaccine program could be offensive BW use. Furthermore, research that increases the virulence of an organism is not necessarily part of an offensive strategy because one can argue that virulence needs to be studied to develop adequate defense.

The key element distinguishing offensive from defensive research is intent. If the intent of the researcher or the goals of the research program is the capability to develop and produce BW, then the research is offensive BW research. If the intent is to have the capability to develop and produce defenses against BW use, then the research is defensive BW research. While it is true that nations may have policies of open disclosures (i.e., no secret research), 'intent' is not observable.

Although the terms 'offensive BW research' and 'defensive BW research' may have some use in describing intent, it is more a philosophical than a practical distinction, one that is based on trust rather than fact.

Secrecy in Biological Warfare-Related Research

Neither the Geneva Protocol nor the 1972 BW Convention prohibits any type of research, secret, or nonsecret. While the BDRP does not conduct secret or classified research, it is possible that secret BW research is being conducted in the United States outside of the structure of the BDRP. The classified nature of the resource material for this work makes it impossible to effectively determine if secret research is being conducted in the United States or any other nation.

It is not, however, unreasonable to assume that other nations conduct significant secret BW research. Therefore, regardless of the facts, one cannot deny the perception that such research exists in a variety of countries and that this perception will exist for the foreseeable future.

Secrecy has been cited as a cause of decreased quality of BW research. If secret research, whether offensive or defensive, is being conducted in the United States or other nations, it is unclear if the process of secrecy affects the quality of the research. If the secret research process consists of a core of highly trained creative and motivated individuals sharing information, the quality of the research may not suffer significantly. It must be stated, however, that secrecy by its very nature will limit input from a variety of diverse observers.

Secrecy may increase the potential for violations of the 1972 BW Convention; however, violations would probably occur regardless of the secrecy of the research. Secrecy in research can certainly lead to infractions against arbitrary rules established by individuals outside of the research group. The secret nature of the research may lure a researcher into forbidden areas. Additionally, those outside of the research group, such as policymakers, may push for prohibited activities if the sense of secrecy prevails. Secrecy also tends to bind those within the secret arena together and tends to enhance their perception of themselves as being above the law and knowing what is 'right'. As in the case of Oliver North and the Iran–Contra affair, those within the group may believe fervently that the rules must be broken for a justified purpose and a mechanism of secrecy allows violations to occur without penalty.

The distrust between nations exacerbates the perceived need for secret research. The animosity between the United States and the Soviet Union during the 1980s fueled the beliefs that secret research leading to violations of the 1972 BW Convention was being conducted in the Soviet Union. As the belligerence of the 1980s fades into the New World Order, the questions will not focus on the Soviet Union as much as on the Middle East and third-world countries. There are factions in the United States that believe strongly that other countries are conducting secret research that will lead to violations of the Convention. There is also a tendency to believe that the secrecy in one's own country will not lead to treaty violations, while the same secret measures in an enemy nation will result in activities forbidden by international law.

The importance of the concept of secrecy in BW research is related to the perception of secrecy and arms control agreements. Regardless of the degree of secrecy in research, if an enemy believes that a nation is pursuing secret research, arms control measures are jeopardized. The reduction of secrecy has been suggested as a tool to decrease the potential for BW treaty violations. A trend toward reducing secrecy in BW research was exemplified by the 1986 Review Conference of the 1972 BW Convention, which resulted in agreements to exchange more information and to publish more of the results of BW research.

Whether or not these measures have any effect on strengthening the 1972 BW Convention remains to be seen.

Organizations and individuals have urged a renunciation by scientists of all secret research and all security controls over microbiological, toxicological, and pharmacological research. This action has been suggested as a means of strengthening the 1972 BW Convention. The belief that microbiologists should avoid secret research is based on the assumption that (1) secret research is of poor quality due to lack of peer review and (2) secrecy perpetrates treaty violations.

While it may be reasonable to expect microbiologists to avoid secret research, it is not realistic. Secrecy is practiced in almost every type of research including academic, military, and especially industrial. Furthermore, there will always be those, within the military and intelligence structures, who believe that at least some degree of secrecy is required for national security.

Secrecy in BW research is a complex issue. The degree to which it exists is unclear. Individuals are generally opposed to secrecy in BW research although other examples of secrecy in different types of research exist and are generally accepted. The effect of the secrecy on the quality of research, the need for the secrecy, and the choice of microbiologists to participate in secret BW research remain unanswered questions.

Problems Relating to Verification

One of the major weaknesses of the 1972 BW Convention has been the lack of verification protocols. Problems with effectively monitoring compliance include the ease of developing BW agents in laboratories designed for other purposes, and the futility of inspecting all technical facilities of all nations. Measures that have been implemented with the goal of monitoring compliance have included (1) open inspections, (2) intelligence gathering, (3) monitor research, (4) use of sampling stations to detect the presence of biological agents, and (5) international cooperation. The progress achieved with the Chemical Weapons Convention has renewed interest in strengthening mechanisms for verification of compliance with the 1972 BW Convention. While this renewed interest in verification of compliance with the emergence of the Commonwealth of Independent States from the old Soviet Union has brought optimism to the verification issue, the reticence of countries such as Iran and North Korea to cooperate with United Nations inspection teams is a reminder of the complexities of international agreements. The examples herein are typical of the many issues attached to the concept of BW.

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Bioluminescence, Microbial

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Defining Statement
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Glossary

bioluminescence The enzymatic production of light by a living organism.

coevolution Reciprocal heritable change in a host and its symbiont.

cospeciation Evolutionary codivergence of a host and its symbiont.

cosymbiosis The presence of two (or more) species of luminous bacteria in the light organ of a fish or squid.

horizontal gene transfer The acquisition and stable incorporation into the genome of an organism of a gene or genes from another species of organism.

luciferase The light-emitting enzyme; in bacteria, a heterodimeric protein that uses oxygen, reduced flavin mononucleotide, and a long-chain fatty aldehyde as substrates to produce blue-green light.

merodiploidy The stable presence in the genome of an organism of two (or more) copies of a gene or genes.

phylogeny The evolutionary relationships among organisms.

psychrotroph An organism with a growth temperature optimum typically below 20 °C.

quorum sensing A gene regulatory mechanism that operates via low molecular weight, membrane-permeant signal molecules in response to a bacterium's local population density.

systematics The area of biology that addresses the taxonomy and phylogeny of organisms.

Abbreviations

AFLP	amplified fragment length polymorphism
AHLs	acyl-homoserine lactones
cAMP	cyclic AMP
CEAs	ciliated epithelial appendages
CRP	cAMP receptor protein

Defining Statement

Bioluminescence is the enzymatic production of light by a living organism. Many different kinds of organisms are bioluminescent. Luminous microbes include some fungi, certain unicellular eukaryotes, and several kinds of bacteria. This article summarizes information on bioluminescence in bacteria, from the perspectives of biochemistry, systematics, ecology and symbiosis, genetics, and evolution.

Introduction

Bioluminescence, the enzyme catalyzed emission of light, is an attribute of many different kinds of organisms. The yellow-green flashes of light made by fireflies at dusk in summer are one of the more commonly observed forms of bioluminescence. Various other terrestrial and many marine organisms are luminescent, including cnidarians, mollusks, annelids, arthropods, echinoderms, and fish. Certain eukaryotic microorganisms, the protist *Gonyaulax*, and certain fungi, for example, also emit light, as do several kinds of bacteria. A common theme in bioluminescence is the use of oxygen as a substrate for the light-emitting enzyme, referred to generically as luciferase. However, in most of these organisms, the substrates that luciferase uses other than oxygen are completely different, and the luciferases themselves exhibit no homology in their nucleotide sequences. The biochemical diversity of extant bioluminescence systems and their lack of DNA sequence homology indicate that bioluminescence has evolved independently many times during the history of life on Earth. This article focuses on the smallest of luminous organisms with presumably the longest evolutionary history, the bioluminescent bacteria. Additional information on bioluminescence in eukaryotic microorganisms can be found in references listed in Further Reading.

Luminous bacteria (**Table 1**) are those bacteria whose genomes naturally contain genes for bacterial luciferase and for the enzymes that produce a long-chain aldehyde substrate used by luciferase in light emission. Bacterial luminescence and many of the luminous bacteria themselves have been known for some time. During the 1700s and 1800s, various animal products, such as meats, fish, and eggs, the decaying bodies of marine and terrestrial animals, and even human wounds and corpses, were seen to emit light. Those observations were preceded many years by the demonstration of Robert Boyle in 1668 that the 'uncertain shining of Fishes', the light coming from decaying fish, required air, long before the existence of bacteria was known. Light-emitting bacteria were first isolated from nature in the 1880s by the early microbiologists Bernard Fischer and Martin Beijerinck, and they have been subjects of biochemical, physiological, ecological, and, more recently, genetic studies since that time. Although luminous bacteria are in most ways not different physiologically and genetically from other bacteria, studies of these bacteria from the perspective of their light-emitting capability have led to substantial progress in understanding quorum sensing in bacteria, now a major research theme in microbiology, and in understanding the ecology and genetics of mutualistic bacterial symbioses with animals. More recently, studies of these bacteria using modern phylogenetic approaches, while opening up their evolutionary history, have made contributions to understanding the nature of bacterial species and to bacterial biogeography.

Biochemistry of Bacterial Light Production

Light emission in bacteria (**Figure 1**) is catalyzed by bacterial luciferase, a heterodimeric protein of approximately 80 kDa, composed of an α-subunit (40 kDa) and a β-subunit (37 kDa). Bacterial luciferase mediates the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (RCHO) by O_2 to produce blue-green light, according to the following reaction:

$$\mathrm{FMNH_2} + \mathrm{O_2} + \mathrm{RCHO} \xrightarrow{\text{luciferase}} \mathrm{FMN} + \mathrm{H_2O} + \mathrm{RCOOH} + \text{light } (\sim 490\,\text{nm})$$

In the luminescence reaction, binding of $FMNH_2$ by the enzyme is followed by interaction with O_2 and then binding of aldehyde, forming a highly stable enzyme/substrate complex that slowly decays with the oxidation of the $FMNH_2$ and aldehyde substrates and the emission of light. Quantum yield for the reaction has been estimated at 0.1–1.0 photons. The reaction is highly specific for $FMNH_2$, and the aldehyde substrate *in vivo* is likely to

Table 1 Species and habitats of luminous bacteria[a]

Species	*Habitats*[b]
Aliivibrio fischeri	Coastal seawater, light organs of monocentrid fish and sepiolid squids
Aliivibrio logei	Coastal seawater, light organs of sepiolid squids
Aliivibrio salmonicida	Tissue lesions of Atlantic salmon
Photobacterium kishitanii	Skin and intestines of marine fishes, light organs of deep sea fishes
Photobacterium leiognathi	Coastal seawater, light organs of leiognathid fishes and loliginid squids
Photobacterium mandapamensis	Coastal seawater, light organs of apogonid and leiognathid fishes
Photobacterium phosphoreum	Skin and intestines of marine fishes, coastal and open ocean seawater
Photorhabdus asymbiotica	Human skin lesions
Photorhabdus luminescens	Insect larvae infected with heterorhabditid nematodes
Photorhabdus temperata	Insect larvae infected with heterorhabditid nematodes
Shewanella hanedai	Seawater and sediment
Shewanella woodyi	Seawater and ink sac of squid
Vibrio chagasii	Coastal seawater
Vibrio cholerae	Coastal seawater, brackish and estuarine waters
Vibrio damselae	Coastal seawater
Vibrio harveyi	Coastal seawater and sediments
Vibrio orientalis	Coastal seawater, surfaces of shrimp
Vibrio splendidus	Coastal seawater
Vibrio vulnificus	Coastal seawater, oysters

[a] All are members of *Gammaproteobacteria*, phylum *Proteobacteria*, domain *Bacteria*.

[b] Listed are typical habitats from which luminous strains of the species have been isolated.

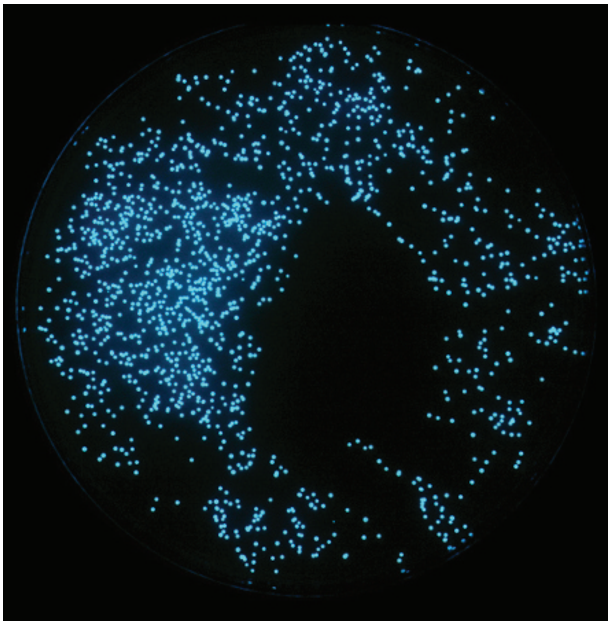

Figure 1 Bacterial bioluminescence. Cells of *Photobacterium kishitanii*, a newly described species of luminous bacteria, have formed colonies on this plate of nutrient seawater agar. The bacteria were taken from the ventral light organ of the deep sea fish *Chlorophthalmus albatrossis* (Chlorophthalmidae). The plate was photographed in the dark by the light the bacteria produce.

be tetradecanal. Synthesis of the long-chain aldehyde and its recycling from the long-chain fatty acid are catalyzed by a fatty acid reductase complex composed of three polypeptides, an NADPH-dependent acyl protein reductase (54 kDa), an acyl transferase (33 kDa), and an ATP-dependent synthetase (42 kDa). The activity of this complex is essential for the production of light in the absence of exogenously added aldehyde.

The genes for bacterial light production are present as a coordinately expressed set of genes, *luxCDABEG*, which is the *lux* operon (**Figure 7**). The *luxA* and *luxB* genes encode the α- and β-subunits of luciferase, *luxC, luxD*, and *luxE* encode the polypeptides of the fatty acid reductase complex, and *luxG* encodes a flavin reductase. The *luxCDABE* genes are present and have the same gene order in all luminous bacteria examined to date, a defining characteristic of these organisms. More information on the biochemistry of bacterial light production can be found in the references listed in Further Reading.

Species and Systematics of Luminous Bacteria

At present, 19 species of luminous bacteria have been identified (**Table 1**). This list includes some species in which only certain strains are luminous. It should be noted that many more kinds of luminous bacteria remain to be discovered and characterized. The basis for this statement is the recent identifications of new species of luminous bacteria (i.e., *Photobacterium kishitanii*) and of luminous strains of species not previously known to be luminous (i.e., *Aliivibrio salmonicida*), as well as descriptions of new species in progress at this time and not listed in Table 1.

Luminous bacteria are all Gram-negative, nonsporeforming, motile, chemoorganotrophs. Most, that is, those in genera *Vibrio, Aliivibrio, Photobacterium*, and *Photorhabdus*, are facultatively aerobic, able to use oxygen in respiration and also able to use sugars by fermentation for energy generation when oxygen and other suitable terminal electron acceptors are not available. In contrast, *Shewanella* species use only the respiratory mode of energy generation. Luminous *Vibrio, Aliivibrio, Photobacterium*, and *Shewanella* species are found in the marine environment, whereas *Photorhabdus* species are terrestrial. Luminous strains of *Vibrio cholerae* have been isolated from coastal seawater as well as from brackish and freshwater environments. Some species of luminous bacteria form highly specific, mutually beneficial bioluminescent symbioses with marine fishes and squids (discussed below).

Luminous bacteria are members of three families, *Vibrionaceae, Shewanellaceae*, and *Enterobacteriaceae*, all of which belong to *Gammaproteobacteria*, a class in phylum Proteobacteria of domain *Bacteria*. A phylogeny of luminous bacteria is shown in **Figure 2**. This phylogeny is based on sequences of the 16S rRNA gene, which encodes the RNA component of the small subunit of the bacterial ribosome, and *gyrB*, a housekeeping gene that encodes DNA gyrase subunit B and that, like the 16S rRNA gene, is useful for evolutionary analysis of bacteria. The figure reveals that most species of luminous bacteria are members of the *Vibrionaceae* genera *Vibrio, Photobacterium*, and *Aliivibrio*. Species previously known as members of the *Vibrio fischeri* species group, that is, *V. fischeri, Vibrio logei, Vibrio salmonicida*, and *Vibrio wodanis*, were for many years known to differ from members of *Vibrio* and *Photobacterium.* These bacteria have been reclassified in accord with those differences as members of a new genus, *Aliivibrio.* Four species of this genus are currently recognized, namely, *Aliivibrio fischeri, Aliivibrio logei, A. salmonicida*, and *Aliivibrio wodanis.* The phylogenetic tree includes all the luminous bacteria but only a few representative nonluminous species in *Vibrionaceae, Shewanellaceae*, and *Enterobacteriaceae*; luminous bacteria make up only a small number of the many species in these genera. Within *Vibrio, Aliivibrio, Photobacterium*, and *Shewanella*, there are many species of nonluminous bacteria; in contrast, genus *Photorhabdus* as presently defined contains only luminous species. Even species characterized as luminous, such as *Photorhabdus luminescens*, can contain strains that do not produce light,

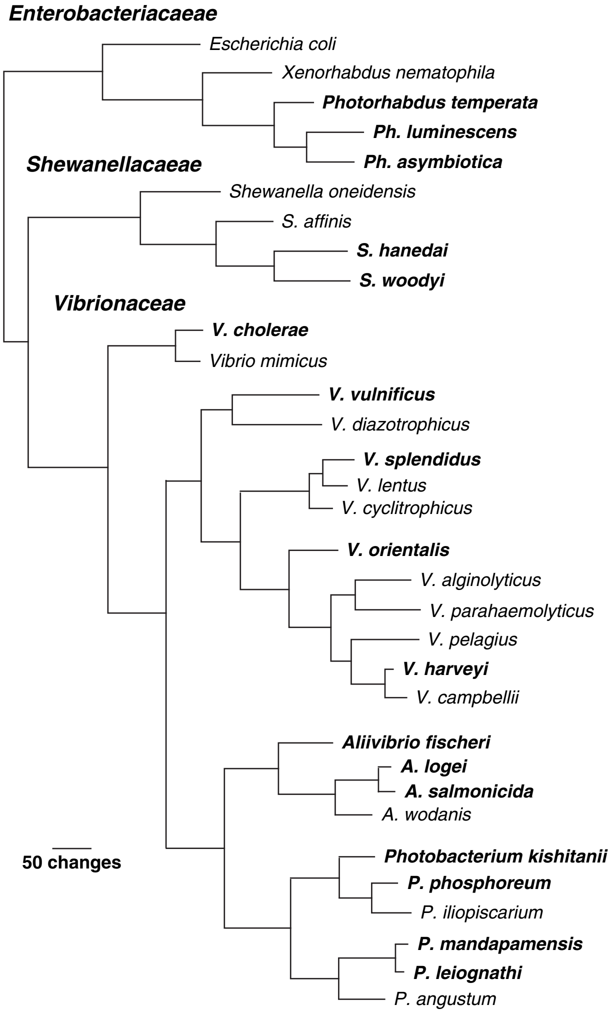
Enterobacteriaceae
Escherichia coli
Xenorhabdus nematophila
Photorhabdus temperata
Ph. luminescens
Ph. asymbiotica
Shewanellacaeae
Shewanella oneidensis
S. affinis
S. hanedai
S. woodyi
Vibrionaceae
V. cholerae
Vibrio mimicus
V. vulnificus
V. diazotrophicus
V. splendidus
V. lentus
V. cyclitrophicus
V. orientalis
V. alginolyticus
V. parahaemolyticus
V. pelagius
V. harveyi
V. campbellii
Aliivibrio fischeri
A. logei
A. salmonicida
A. wodanis
50 changes
Photobacterium kishitanii
P. phosphoreum
P. iliopiscarium
P. mandapamensis
P. leiognathi
P. angustum

Figure 2 Phylogeny of luminous bacteria. This analysis (provided by Dr. Jennifer Ast, University of Michigan) is based on sequences of the 16S rRNA and *gyrB* genes. Luminous species (in boldface) are found in three families, *Vibrionaceae, Shewanellaceae*, and *Enterobacteriaceae*. These families contain many more nonluminous species than shown here.

and some species, such as *V. cholerae* and *Vibrio vulnificus*, contain relatively few strains that are luminous. The phylogenetic relationships among luminous bacteria and the presence of luminous and nonluminous species in several groups provide insights into the evolution of the bacterial luminescence system, a topic discussed in a later section.

Our knowledge of what species of bacteria are luminous is based to a large extent on the production of high levels of light by many commonly encountered luminous species. This criterion, however, does not recover all luminous bacteria. The problem is that some luminous bacteria produce a high level of light under natural conditions but little or no light when grown on laboratory media; 'cryptically luminous' bacteria therefore can be missed in screening environmental samples for light-emitting bacteria. Examples include luminous bacteria infecting crustaceans and strains of *A. fischeri* symbiotic with the Hawaiian sepiolid squid, *Euprymna scolopes*. Other examples are *V. cholerae*, many strains of which carry *lux* genes but produce little or no light, and *A. salmonicida*, which requires addition of aldehyde to stimulate light production. Enzyme assay and antibody methods previously were used to indicate the presence of luciferase in several nonluminous *Vibrio* spp., and *lux* gene-containing bacteria not producing light in culture have been identified with *luxA*-based DNA probes from various seawater samples. A further complication is that luminescence often is not phenotypically stable; strains that are luminous on primary isolation often become dim or dark in laboratory culture and therefore may be discarded as nonluminous contaminants. It is therefore reasonable to assume that more luminous bacteria exist than are listed here (**Table 1**). Supporting this view, descriptions of additional new luminous bacteria and of bacterial species newly recognized to contain luminous strains are underway at this time.

Methods for taxonomic identification of luminous bacteria have changed significantly over the past few years, with more emphasis now being placed on DNA sequence-based phylogenies over previously standard descriptive phenotypic and genotypic methods. Analysis of *lux* gene sequences together with sequences of housekeeping genes, such as the16S rRNA gene, *gyrB, pyrH, recA, rpoA*, and *rpoD*, has proven helpful both for defining evolutionary relationships among luminous bacteria and for the identification of new species. For example, bacteria previously grouped as members of *Photobacterium phosphoreum* based on phenotypic and genotypic traits have been resolved by a multigene phylogenetic approach as three distinct species (**Figure 3**). The more definitive species-level resolution provided by molecular phylogenetic analysis is proving to be helpful also in opening up the species-specific ecologies of luminous bacteria.

Habitats and Ecology of Luminous Bacteria

Marine

Luminous bacteria are globally distributed in the marine environment (**Table 1**) and can be isolated from seawater, sediment, and suspended particulates. They also colonize the skin of marine animals as saprophytes, their intestinal tracts as commensal enteric symbionts, and their tissues and body fluids as parasites, where they presumably use the organic compounds available in these habitats as sources of energy and carbon. Certain of the luminous bacteria form highly specific bioluminescent symbioses with marine fish and squids. They also colonize marine algae, but agar-digesting luminous bacteria are rare.

In seawater and marine sediments and on the skin of marine animals, luminous bacteria are a consistent but

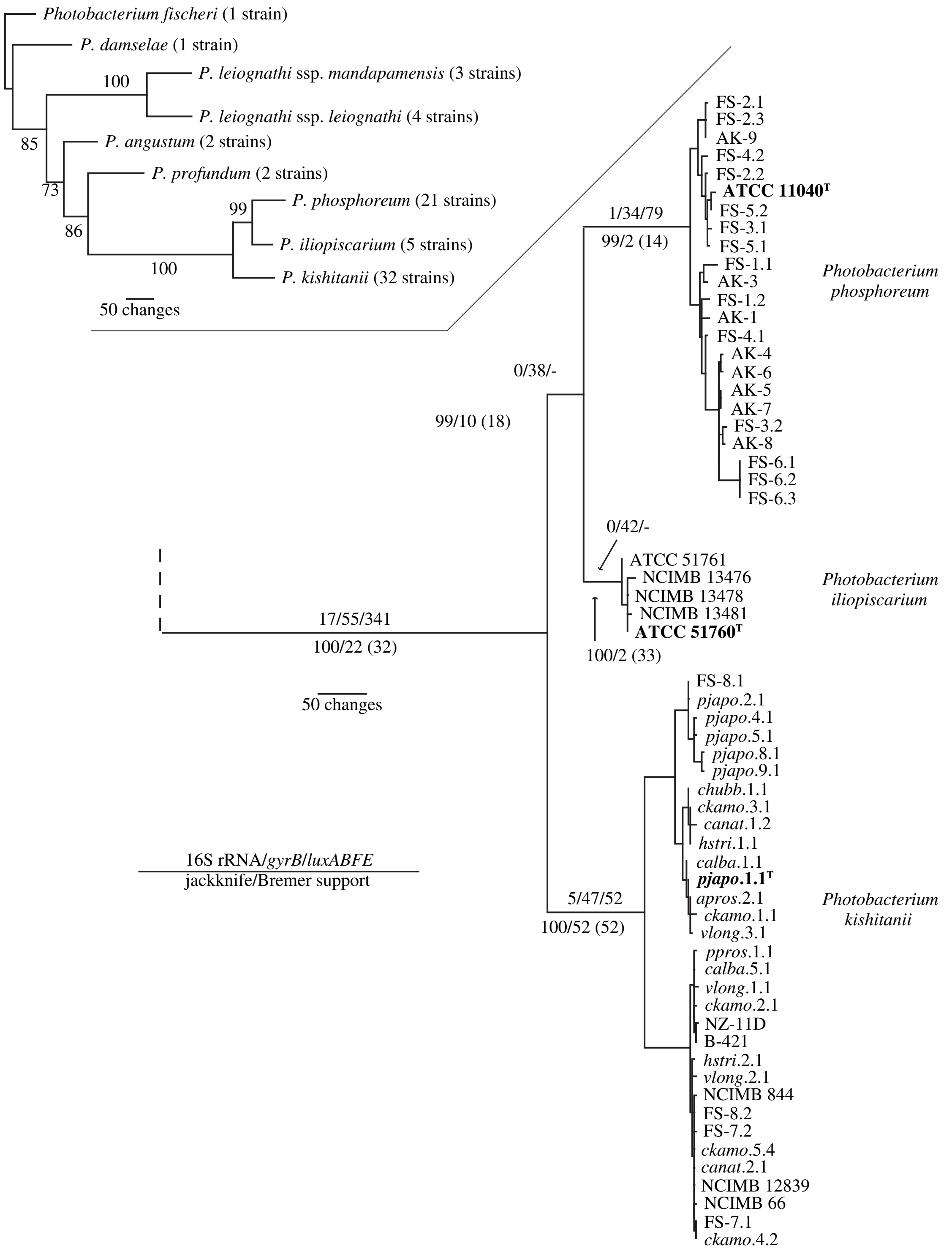
Photobacterium fischeri (1 strain)
P. damselae (1 strain)
100
P. leiognathi ssp. mandapamensis (3 strains)
P. leiognathi ssp. leiognathi (4 strains)
85
P. angustum (2 strains)
73
P. profundum (2 strains)
99
P. phosphoreum (21 strains)
86
P. iliopiscarium (5 strains)
100
P. kishitanii (32 strains)
50 changes
FS-2.1
FS-2.3
AK-9
FS-4.2
FS-2.2
ATCC 11040T
FS-5.2
FS-3.1
FS-5.1
FS-1.1
AK-3
FS-1.2
AK-1
FS-4.1
AK-4
AK-6
AK-5
AK-7
FS-3.2
AK-8
FS-6.1
FS-6.2
FS-6.3
1/34/79
99/2 (14)
Photobacterium phosphoreum
0/38/-
99/10 (18)
0/42/-
ATCC 51761
NCIMB 13476
NCIMB 13478
NCIMB 13481
ATCC 51760T
100/2 (33)
Photobacterium iliopiscarium
17/55/341
100/22 (32)
50 changes
FS-8.1
pjapo.2.1
pjapo.4.1
pjapo.5.1
pjapo.8.1
pjapo.9.1
chubb.1.1
ckamo.3.1
canat.1.2
hstri.1.1
calba.1.1
pjapo.1.1T
apros.2.1
ckamo.1.1
vlong.3.1
16S rRNA/gyrB/luxABFE
jackknife/Bremer support
5/47/52
100/52 (52)
Photobacterium kishitanii
ppros.1.1
calba.5.1
vlong.1.1
ckamo.2.1
NZ-11D
B-421
hstri.2.1
vlong.2.1
NCIMB 844
FS-8.2
FS-7.2
ckamo.5.4
canat.2.1
NCIMB 12839
NCIMB 66
FS-7.1
ckamo.4.2

Figure 3 Phylogenetic resolution of the members of the *Photobacterium phosphoreum* species group. The phylogenies shown are based on combined analysis of the 16S rRNA, *gyrB*, and *luxABFE* genes. The numbers above the branches represent the steps each locus (16S rRNA gene, *gyrB* and *luxABFE*) contributed to branch length. The 16S rRNA gene contributed several steps (17 steps) to the separation of the *P. phosphoreum* group from the rest of *Photobacterium*, but few steps (0–1 step) to the branches separating *P. phosphoreum* and *Photobacterium iliopiscarium*; the sequences of *Photobacterium kishitanii* strains differed slightly (five steps) from those of *P. phosphoreum* and *P. iliopiscarium*. In contrast, the much greater sequence divergence of the *gyrB* and *luxABFE* genes permits resolution of *P. phosphoreum, P. iliopiscarium*, and *P. kishitanii* as separate species. Numbers below the branches represent jackknife resampling values and Bremer support values. Species type strains are in boldface. Reproduced from Ast JC and Dunlap PV (2005) Phylogenetic resolution and habitat specificity of the *Photobacterium phosphoreum* species group. *Environmental Microbiology* 7: 1641–1654.

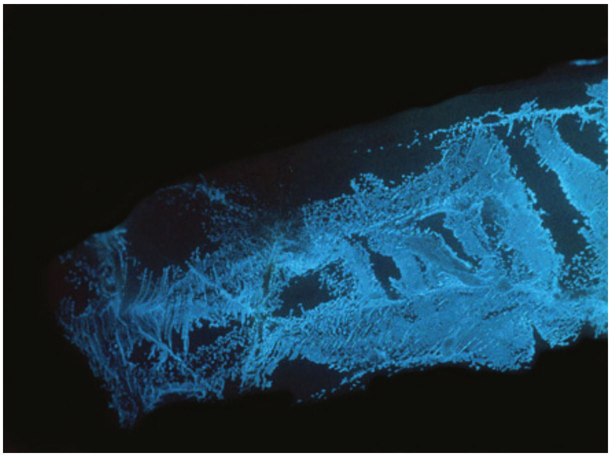

Figure 4 Saprophytic growth of luminous bacteria. Luminous bacteria have colonized this slice of fish meat, which was photographed in the dark by the light the bacteria produce. Cells of luminous bacteria injected into the flank muscle tissue of live fish can rapidly reproduce and cause septicemia and death of the fish, indicating the potential of luminous bacteria for pathogenesis.

usually small fraction of the bacteria present. Also in the intestinal tracks of marine fishes and other animals, luminous bacteria usually make up a small but consistent percentage of the bacteria present than can grow under aerobic laboratory conditions, although in some cases, luminous colonies have been observed to form 50% or more of the colonies arising on plates of seawater-based complete medium inoculated with intestinal contents. Luminous bacteria attain exceptionally high numbers, up to 10^9 to 10^{11} cells per ml, in light organs of fishes and squids, where they exist in mutualistic symbioses, and as incidental parasites reproducing in the hemocoel of marine crustaceans and other marine animals. Luminous bacteria grow readily, for example, on fish muscle tissue (**Figure 4**). Reproduction in each of these habitats releases cells into seawater, providing inocula for colonization of the other habitats. In colonizing free-living, saprophytic, commensal, and parasitic habitats, luminous bacteria coexist and compete for nutrients with many other kinds of bacteria. The exception to their existence as members of a diverse community of bacteria is bioluminescent symbiosis; light organs of fishes and squids are highly specific to certain species of luminous bacteria and do not harbor nonluminous species.

The distributions of individual species of luminous bacteria and their numbers in a given habitat correlate with certain environmental factors, as is seen for nonluminous bacteria. Primary among these factors are temperature and depth in the marine environment, nutrient limitation, and sensitivity to solar irradiation induced photooxidation in surface waters. Temperature, along with being an important environmental factor, can influence whether luminous bacteria are detected from environmental samples. For example, *Shewanella hanedai* and some strains of *A. logei*, which are psychrotrophic, grow and produce light at low temperature (e. g., 15 °C), and grow but do not produce light at room temperature (24 °C). Therefore, incubation of platings of environmental samples at the lower temperatures may reveal the presence of other psychrotropic luminous species. Temperature relationships of luminous bacteria appear to be species-specific. For example, *S. woodyi*, characterized from squid ink and seawater in the Alboran Sea near Gibraltar and *A. fischeri*, species that are closely related to *S. hanedai* and *V. logei*, respectively, grow and produce light at room temperature. Some bacteria appear to be eurythermal, growing and producing light from low to relatively high temperatures.

Freshwater

V. cholerae apparently is the only luminous bacterium to have been isolated from freshwater and brackish estuarine habitats. A luminous non-O1 strain of this species, sometimes called *Vibrio albensis*, was isolated from the Elbe River in Germany in 1893, and non-O1 strains of *V. cholerae* sometimes have been isolated from diseased, glowing crustaceans from freshwater lakes.

Terrestrial

Luminous bacteria are found also in the terrestrial environment, where they infect a variety of terrestrial insects, causing the infected animal to glow. These bacteria presumably are members of *P. luminescens* and *Photorhabdus temperata*, which occur also as the mutualistic symbionts of nematodes, which are common in soil, or *Photorhabdus asymbiotica*, which has been isolated from human clinical infections initiated by spider and insect bites (**Table 1**).

P. luminescens and *P. temperata* occur as the mutualistic symbionts of entomopathogenic nematodes of the family Heterorhaditidae. They are carried in the intestine of the infective juvenile stage of the nematode, contribute to a lethal infection of insect larvae, and permit completion of the nematode life cycle. When the nematode enters the insect, via the digestive tract or other openings, and penetrates the insect's hemocele, the bacteria are released into the hemolymph, constituents of which they utilize for growth. The bacteria elaborate a variety of extracellular enzymes that presumably breakdown macromolecules of the hemolymph. Proliferation of the bacteria leads to death of the insect, the carcass of which can become luminous. Through consumption of the bacteria or products of bacterial degradation of the hemolymph, the nematodes develop and sexually reproduce. Completion of the nematode life cycle involves reassociation with the bacteria and the emergence from the insect cadaver of the nonfeeding infective juveniles, the intestines of which are colonized by the bacteria. Cells of *P. luminescens* presumably are present in soil, but association with the nematode may be necessary for their survival and dissemination. Luminescence of the infected insect larva might function to attract nocturnally active animals to feed on the glowing carcass, thereby increasing the opportunities for the bacterium and the nematode to be disseminated. However, luminescence is not required for successful symbiosis with the nematode; not all strains of *P. luminescens* produce luminescence. Furthermore, bacteria in the genus *Xenorhabdus*, which are symbiotic with entomopathogenic nematodes in the family Steinernematidae, are ecologically very similar to *Photorhabdus*, except that they do not produce light. The similarities between the life styles and activities of *Photorhabdus* and *Xenorhabdus* might be a case of ecological convergence.

Bioluminescent Symbiosis

A different kind of symbiotic association is seen with members of several families of marine fishes and squids, which form mutually beneficial associations with luminous bacteria (**Figure 5**). These associations, of which there are many, are called bioluminescent symbioses. The bacteria are housed extracellularly in the body of the animal in a complex of tissues called a light organ. In fishes, the light organs are outpocketings of the gut tract (most fishes), or are positioned below the eyes (anomalopids), in the lower jaw (monocentrids), or at the tips of tissue extensions (ceratioids). In squids, they are found as bilobed structures within the mantle cavity, associated with the ink sac. Accessory tissues associated with the light organ, that is, lens, reflector and light-absorbing barriers, direct and focus the light the bacteria produce. The animal uses the bacterial light in various luminescence displays that are associated with sex-specific signaling, predator avoidance, locating or attracting prey, and schooling. In turn, the bacteria, which are present at a high cell density (**Figure 6**), use nutrients obtained from the host to reproduce and are disseminated from the animal's light organ into the environment.

Five species of marine luminous bacteria have been identified as light-organ symbionts, *A. fischeri* (previously *V. fischeri*), *A. logei* (previously *V. logei*), *Photobacterium leiognathi, P. kishitanii*, and *Photobacterium mandapamensis*. The families of squids and fishes whose light organs these bacteria colonize are listed in **Table 2**. *P. phosphoreum* has not been found as a light-organ symbiont, whereas *P. kishitanii*, a newly identified species closely related to *P. phosphoreum*, is found in the light organs of a wide variety of deep, cold-dwelling fishes. Luminous bacterial symbionts of two groups of fishes, the flashlight fish, family Anomalopidae, and deep sea anglerfish, for example, families Melanocetidae and Ceratiidae in suborder Ceratioidei, have not yet been brought into laboratory culture and therefore remain to be identified; these as-yet uncultured bacteria probably represent new species. In the cases studied, the newly hatched, aposymbiotic animals acquire their symbiotic bacteria from the environment with each new host generation.

Based on symbiont acquisition from the environment, the association between the sepiolid squid *E. scolopes* and the *A. fischeri* has emerged as an experimental system with which examine various aspects of the association. The nascent, rudimentary light organ lobes in aposymbiotic hatchling juvenile squids bear a pair of ciliated epithelial appendages (CEAs) and contain three simple sac-like epithelial tubules embedded in the undifferentiated accessory tissues. The outer portions of these tubules are ciliated and directly connect to the mantle cavity via a lateral pore. Colonization of the epithelial tubules, which is facilitated by ciliary beating of the CEAs, occurs through these pores, which later coalesce, with the formation of a ciliated duct for each light organ lobe. Notably, colonization triggers regression of the CEAs within a few days. Other morphological changes upon colonization include alterations in the epithelial cells of the distal portions of the light organ tubules, which develop a dense microvillous brush border. Nonetheless, the developmental program giving rise to the light organ and accessory tissues runs independently of the presence of the bacteria; these tissues develop normally in aposymbiotic animals, and the light organ can remain receptive to colonization in aposymbiotic animals from hatching to adulthood. At the level of bacterial genes and functions necessary for symbiosis, motility of *A. fischeri*, via polar flagella, is necessary for colonization of the squid light organ. Various other bacterial genes may be involved in the ability of this bacterium to establish bioluminescent symbiosis with *E. scolopes*.

Bioluminescent symbiosis is a special class of symbiosis, different in fundamental ways from other kinds of symbiotic associations. In most bacterial mutualisms with animals and plants, the host is dependent nutritionally on its symbiotic bacteria, for bacterial fixation of carbon or nitrogen, the activity of bacterial extracellular degradative enzymes such as cellulases, or bacterial provision of vitamins or other essential nutrients to the host. The absence of symbiotic bacteria consequently can have a profound effect on the growth, development, and survival of the host. In the bioluminescent symbiosis of *A. fischeri* and the sepiolid squid *E. scolopes*, however, animals cultured aposymbiotically from hatching to adulthood grow, develop, and survive in the laboratory as well as animals colonized by *A. fischeri*. This observation, which indicates that, at least under laboratory conditions, the animal is not dependent on the bacterium for completion of its life cycle, suggests that *A. fischeri* makes no major nutritional contribution to the animal. The metabolic dependency of *E. scolopes* on *A. fischeri* probably is limited to light production, and selection

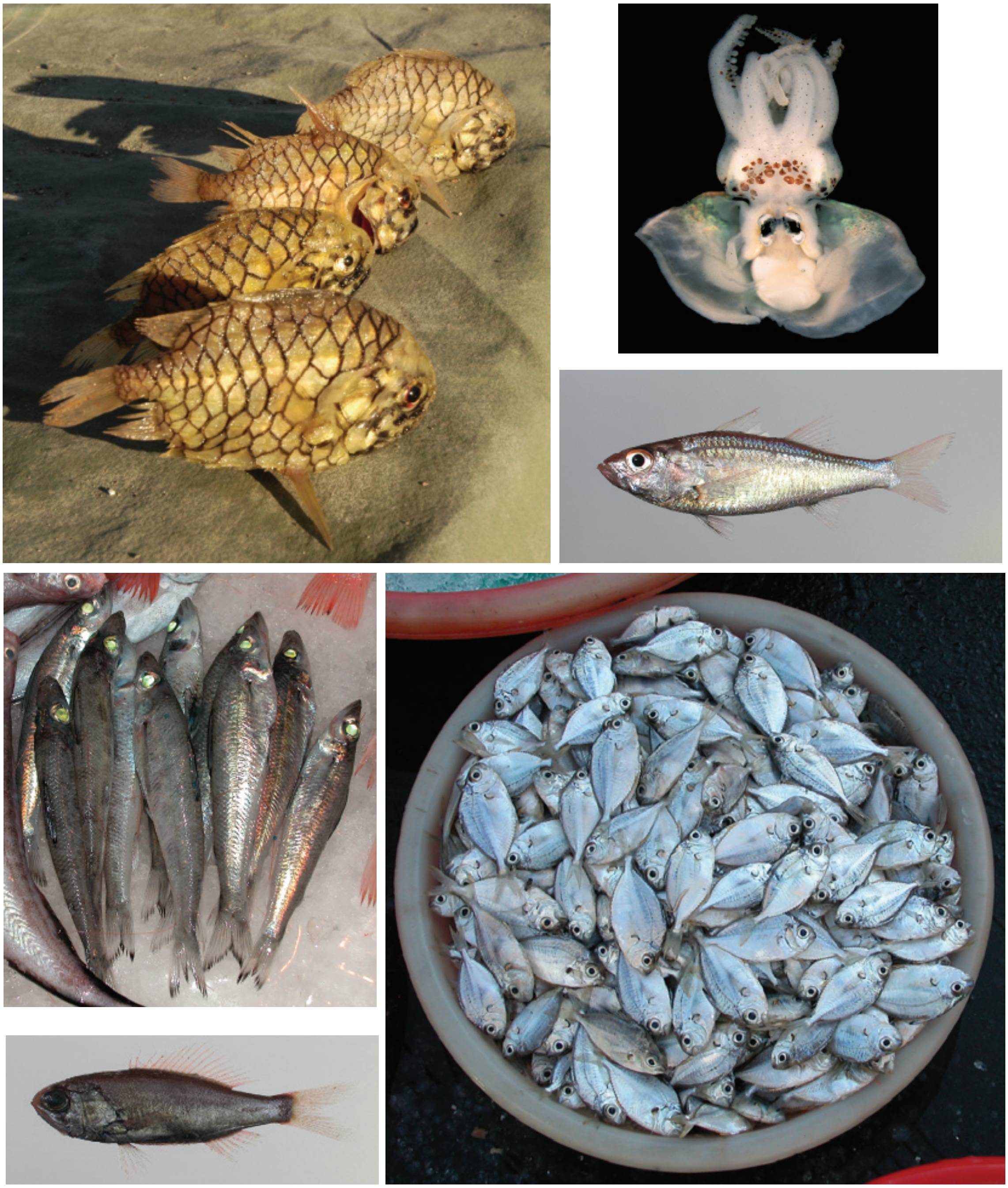

Figure 5 Luminous animals. Different fish and squid hosts of light-organ symbiotic luminous bacteria are shown. Counterclockwise from the upper left, the animals are *Monocentris japonicus* (Monocentridae), host of *Aliivibrio fischeri*; *Chlorophthalmus albatrossis* (Chlorophthalmidae), host of *Photobacterium kishitanii*; *Aulotrachichthys prosthemius* (Trachichthyidae) (photo provided by Atsushi Fukui, Tokai University), host of *P. kishitanii*; *Leiognathus splendens* (Leiognathidae), host of *Photobacterium leiognathi*; *Acropoma japonicus* (Acropomatidae) (photo provided by Atsushi Fukui, Tokai University), host of *Photobacterium mandapamensis*; and, *Euprymna scolopes* (Sepiolidae) (dissected to reveal bilobed light organ in this ventral view), host of *A. fischeri*.

that maintains the association presumably is ecological, not nutritional, at the level of the squid's ability to use light to avoid predators.

Bioluminescent symbioses differ in this and other ways also from endosymbiotic associations, which are mutually obligate relationships in which the symbiotic bacteria are housed intracellularly and are transferred maternally. Symbiotic luminous bacteria are housed extracellularly, and in most cases they are known not to be obligately dependent on the host for their reproduction. Unlike obligate intracellular bacteria, the symbiotic luminous colonize a variety of other marine habitats, including

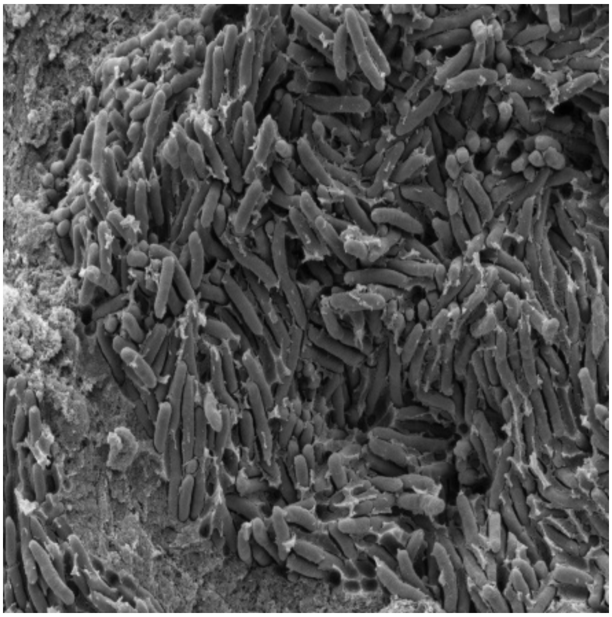

Figure 6 Symbiotic light-organ bacteria. This scanning electron micrograph of a section of the light organ of the fish *Siphamia versicolor* (Apogonidae) (micrograph prepared by Sasha Meshinchi, Microscopy and Imaging Laboratory, University of Michigan) shows the exceptionally high density of bacteria that is typical of bioluminescent symbioses.

intestinal tracts, skin, and body fluids of marine animals, sediment, and seawater, where they coexist and compete with many other kinds of bacteria as members of commensal, saprophytic, pathogenic, and free-living bacterial communities. A second major difference with endosymbiotic associations is that symbiotic luminous bacteria are acquired from the environment with each new generation of the host instead of being transferred vertically through the maternal inheritance mechanisms seen for obligate bacterial endosymbionts of terrestrial and marine invertebrates.

Bioluminescent symbioses also the lack the strict specificity expected for partners in a mutually obligatory, endosymbiotic association. Two or three different species of bacteria colonize the light organs of members of a single fish or squid family (**Table 2**), and even light organs of individual fish and squids can contain two bacterial species, a state called bacterial cosymbiosis. Furthermore, the bacteria resident within individual light organs often represent several genetically distinct strain types. The ability of host animals to accept genetically distinct strain types and even different species of luminous bacteria as symbionts suggests that a strict genetically based selection by a host of its specific symbiont probably is not operative in these associations. An alternative explanation for the patterns of symbiont–host affiliation observed in nature is that the species of luminous bacteria most abundant and active where aposymbiotic hatchling animals first encounter bacteria determines which species of luminous bacteria are most likely to initiate the symbiosis. Temperature, for example, influences the presence and relative numbers of the different species of luminous bacteria in the marine environment. Thus, lower temperatures, found in deeper

Table 2 Squid and fish families harboring light-organ symbiotic luminous bacteria

	Bacterial species					
Host family[a]	Aliivibrio fischeri	Aliivibrio logei	Photobacterium kishitanii	Photobacterium leiognathi	Photobacterium mandapamensis	*Not identified*[b]
Squids						
Loliginidae				+		
Sepiolidae	+	+				
Fishes						
Opisthoproctidae			+			
Chlorophthalmidae			+			
Macrouridae	+		+			
Moridae	+		+		+	
Melanocetidae						+
Ceratiidae						+
Anomalopidae						+
Trachichthyidae			+			
Monocentridae	+					
Acropomatidae			+	+	+	
Apogonidae					+	
Leiognathidae				+	+	

[a] For a listing of the bacteria colonizing individual species of squids and fishes, see Dunlap PV, Ast JC, Kimura S, Fukui A, Yoshino T, and Endo H (2007) Phylogenetic analysis of host-symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics* 23: 507–523.
[b] Not yet in laboratory culture.

waters, favor the incidence of the more psychrotrophic *A. logei* and *P. kishitanii.* Animals whose eggs hatch out in these waters would be more likely to acquire these species as light organ symbiont. Conversely, warmer temperatures, found in temperate and tropical coastal waters, favor the incidence the more mesophilic *A. fischeri, P. leiognathi*, and *P. mandapamensis*; animals whose eggs hatch out in these waters would be more likely to encounter and therefore take up these bacteria as light-organ symbionts. The general depth and temperature distributions of bacterially luminous animals in the marine environment and the lack of strict specificity between hosts and symbionts are consistent with this environmental congruence hypothesis. Nonetheless, the apparently complete absence of nonluminous bacteria from light organs indicates some form of selection, possibly for a bacterial activity associated with luminescence.

Another major difference between bioluminescent symbiosis and endosymbiosis is that luminous bacteria and their host animals show no evidence of cospeciation. Endosymbiosis is generally assumed to involve coevolutionary interactions, reciprocal genetic changes in host and symbiont that result from the obligate and mutual dependence of each partner on the other. One manifestation of coevolution is a pattern of codivergence (i.e., cospeciation) in which the evolutionary divergence of the symbiont follows and therefore reflects that of the host. Detailed molecular phylogenies of bacterially luminous fishes and squids, however, are very different from and do not resemble the phylogenies of their symbiotic light-organ bacteria. This lack of host–symbiont phylogenetic congruence demonstrates that the evolutionary divergence of symbiotic luminous bacteria has occurred independently of the evolutionary divergence of their host animals.

Bioluminescent symbioses therefore appear to represent a paradigm of symbiosis that differs fundamentally from associations involving obligate, intracellular, maternally transferred symbionts. While fishes and squids are dependent ecologically on luminous bacteria, the bacteria are not obligately dependent on their bioluminescent hosts. The evolutionary (genetic) adaptations for bioluminescent symbiosis, that is, presence of light organs that can be colonized by luminous bacteria, accessory tissues for controlling, diffusing and shaping the emission of light, and behaviors associated with light emission, all are borne by the animal. No genetic adaptations have been identified in the bacteria that are necessary for and specific to their existence in light organs compared to the other habitats they colonize. Luminous bacteria therefore seem to be opportunistic colonizers, able to persist in animal light organs as well as in a variety of other habitats to which they are adapted.

Regulation of Bacterial Luminescence

Quorum Sensing

Luciferase synthesis and luminescence are regulated in many luminous bacteria. This regulation has been studied in detail in *A. fischeri* and *V. harveyi.* At low population density, these bacteria produce very little luciferase and light, whereas at high population density, luciferase levels are induced 100- to 1000-fold and light levels increase by 10^3- to 10^6-fold. This population density-dependent induction of luciferase synthesis and luminescence is controlled in part by the production and accumulation in the cell's local environment of small secondary metabolite signal molecules, called autoinducers (acyl-homoserine lactones (AHLs) and other low molecular weight compounds), which function via regulatory proteins to activate or derepress transcription of the *lux* operon. Originally called autoinduction, this gene regulatory mechanism is now referred to as quorum sensing to reflect its relationship with population density. As a mechanism by which a bacterium can detect its local population density, quorum sensing might function as a diffusion sensor, mediating whether or not cells produce extracellular enzymes and other factors for obtaining nutrients, or as a sensor of host association.

Quorum-sensing control of luminescence in *A. fischeri* and *V. harveyi* involves complex regulatory circuits. In *A. fischeri*, quorum-sensing regulatory genes, *luxR* and *luxI*, are associated with the *lux* operon (**Figure 7**). The *luxR* gene, which is upstream of the *lux* operon and divergently transcribed from it, encodes a transcriptional activator protein, LuxR, that binds autoinducer, *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), forming a complex that binds at a site in the *lux* operon promoter and facilitates association of RNA polymerase with the *lux* operon promoter, thereby activating transcription of the genes for light production. The other regulatory gene, *luxI*, is the first gene of the *lux* operon in this species, *luxICDABEG*; it encodes an acyl-homoserine lactone synthase necessary for synthesis of 3-oxo-C6-HSL. Between *luxR* and *luxI* is a regulatory region containing the *luxR* and *lux* operon promoters. According to a simple model for luminescence induction in *A. fischeri*, cells produce a low level of 3-oxo-C6-HSL, which, as a membrane-permeant molecule, diffuses out of the cells and away into the environment. In seawater, for example, where the number of *A. fischeri* cells is low, 3-oxo-C6-HSL would not accumulate, and transcription of the *lux* operon would remain uninduced. However, under conditions where cells can attain a high population density, such as in gut tracts of fishes, light organs of fish or squid, or in the laboratory in batch culture, the local concentration of 3-oxo-C6-HSL can build up, both

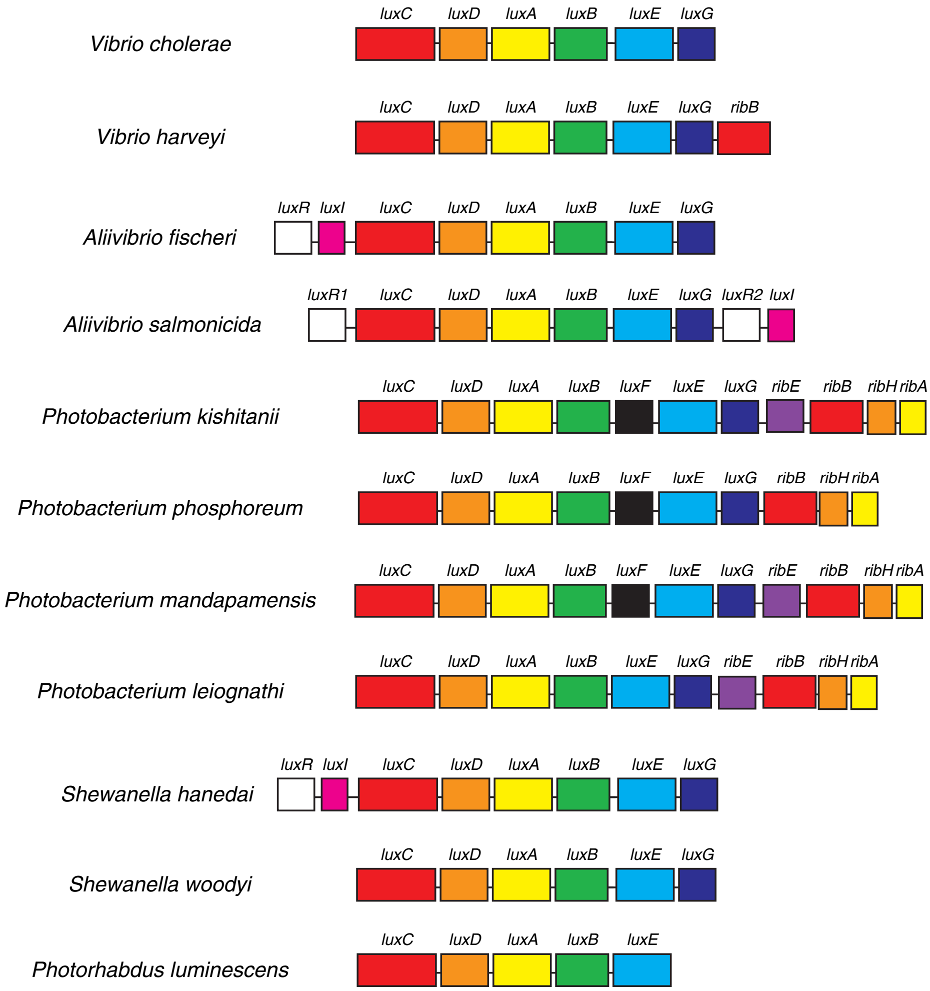
Vibrio cholerae
luxC luxD luxA luxB luxE luxG
Vibrio harveyi
luxC luxD luxA luxB luxE luxG ribB
Aliivibrio fischeri
luxR luxI luxC luxD luxA luxB luxE luxG
Aliivibrio salmonicida
luxR1 luxC luxD luxA luxB luxE luxG luxR2 luxI
Photobacterium kishitanii
luxC luxD luxA luxB luxF luxE luxG ribE ribB ribH ribA
Photobacterium phosphoreum
luxC luxD luxA luxB luxF luxE luxG ribB ribH ribA
Photobacterium mandapamensis
luxC luxD luxA luxB luxF luxE luxG ribE ribB ribH ribA
Photobacterium leiognathi
luxC luxD luxA luxB luxE luxG ribE ribB ribH ribA
Shewanella hanedai
luxR luxI luxC luxD luxA luxB luxE luxG
Shewanella woodyi
luxC luxD luxA luxB luxE luxG
Photorhabdus luminescens
luxC luxD luxA luxB luxE

Figure 7 The *lux* genes of luminous bacteria. Contiguous genes of the luminescence operons of luminous bacteria are aligned to highlight commonalities and differences (Dr. Henryk Urbanczyk and Dr. Jennifer Ast, University of Michigan, assisted in the preparation of this figure). Note that the *lux* operon in *Photobacterium*, referred to as the *lux-rib* operon, contains the *ribEBHA* genes, which are involved in synthesis of riboflavin. Many strains of *Photobacterium leiognathi* carry multiple, phylogenetically distinct *lux-rib* operons.

outside and inside cells. Once 3-oxo-C6-HSL reaches a critical concentration, it then interacts inside the cell with LuxR protein, leading to activation of *lux* operon transcription. Because *luxI* is a gene of the *lux* operon, increased transcription leads to increased synthesis of 3-oxo-C6-HSL, in a positive feedback manner. The result is a rapid and strong induction of luciferase synthesis and luminescence.

Many other regulatory components contribute to quorum sensing in *A. fischeri*. These include: GroEL, which is necessary for production of active LuxR; 3′:5′-cyclic AMP (cAMP) and cAMP receptor protein (CRP), which activate transcription of *luxR* and thereby potentiate the cell's response to 3-oxo-C6-HSL; a second autoinducer, octanoyl-HSL, which is dependent on the *ainS* gene for its synthesis and which interacts with LuxR apparently to delay *lux* operon induction; and several proteins homologous to components of the phosphorelay signal transduction system that controls luminescence in *V. harveyi* (see below). In *A. fischeri* LuxR and 3-oxo-C6-HSL also control several genes unrelated to luminescence, forming a quorum-sensing regulon in this species.

In *V. harveyi*, the quorum-sensing regulatory mechanism differs substantially from that of *A. fischeri*. A transcriptional activator, called LuxR (which is not homologous to *A. fischeri* LuxR) is necessary for *lux* operon transcription, but the regulatory genes controlling that transcription are not contiguous with the *lux* operon, for example. Expression of the *lux* operon in *V. harveyi* is regulated by a quorum-sensing phosphorelay signal transduction mechanism. The mechanism involves two separate two-component phosphorelay paths, each involving a transmembrane sensor/kinase, LuxN and LuxQ, responsive to a separate quorum-sensing signal. The *luxLM* genes are necessary for synthesis of the *N*-3-hydroxy-butanoyl-HSL (3-OH-C4-HSL) signal. In the absence of 3-OH-C4-HSL, LuxN operates as a kinase, phosphorylating LuxU, a signal integrator, which in turn passes the phosphate on to LuxO, which in phosphorylated form is a repressor of the *lux* operon. In the presence of 3-OH-C4-HSL, the activity of LuxN is shifted from kinase to phosphatase, which draws phosphate from LuxU and thereby from LuxO, which then no longer represses *lux* operon expression. A similar activity is carried out by a second signal, AI-2, identified as furanone borate diester, which requires LuxS for its production. AI-2 operates via LuxP, a putative periplasmic protein, to mediate the kinase/phosphatase activity of LuxQ, which in turn, like LuxN, feeds phosphate to or draws it from LuxO. Previously thought to directly repress *lux* operon expression, LuxO may operate indirectly, by controlling a negative regulator of luminescence. Expression of *luxO* itself is subject to repression by LuxT. In a manner possibly analogous to LuxR in *V. fischeri*, LuxR in *V. harveyi* is autoregulatory and responsive to 3-OH-C4-HSL.

Despite the major differences in the quorum-sensing systems of *A. fischeri* and *V. harveyi*, there are several commonalities. These include homologues in *A. fischeri* of the *V. harveyi luxR* gene (*litR*), *luxO, luxU*, and *luxM* (*ainS*), among others. These homologies indicate that a phosphorelay system is likely to be a part of the quorum-sensing system of *A. fischeri*.

Physiological Control

Induction of luciferase synthesis and luminescence are also influenced by physiological factors. Oxygen, amino acids, glucose, iron and osmolarity have distinct effects, depending on the species studied. Those factors that stimulate growth rate of the bacterium, such as readily metabolized carbohydrates, tend to decrease light production and luciferase synthesis. They do so presumably by causing oxygen and reducing power ($FMNH_2$) to be directed away from luciferase and by lowering cellular levels of cAMP and CRP, which are needed to activate *lux* gene expression. In *A. fischeri*, the *lux* regulatory region between *luxR* and *luxICDABEG* contains a cAMP-CRP-binding site, and in *V. harveyi*, a cAMP-CRP-binding site is upstream of *luxCDABEGH*. Conversely, factors that restrict growth rate, such as limitation for iron and either high or low salt concentrations, depending on the species, tend to stimulate the synthesis and activity of luciferase. Much remains to be learned about how these factors operate and the relationship between growth physiology of the cell and regulatory elements controlling *lux* gene expression.

Over the past 25 years, there has been a rapid accumulation of information on how cells regulate luminescence by quorum sensing. Based on these studies, quorum sensing systems biochemically and genetically homologous to that in luminous bacteria have also been identified in a wide variety of nonluminous bacteria, in which quorum sensing controls many cellular activities other than light production, particularly the production of extracellular enzymes and other extracellular factors thought to be useful for bacteria at high population density and in host association. More details on quorum sensing in luminous and nonluminous bacteria are provided in Quorum-Sensing in Bacteria.

Functions of Luminescence in Bacteria

The production of light consumes a substantial amount of energy, through the synthesis of Lux proteins and their enzymatic activity. This energetic cost, which may account for the fact that luminescence is regulated, suggests that activity of the luminescence system plays an important role in the physiology of luminous bacteria. Most attention to what that role might be has focused on oxygen. One possibility is that the light-emitting reaction arose evolutionarily as a detoxification mechanism, removing oxygen and thereby allowing otherwise anaerobic organisms to survive. A related possibility is that luciferase, as an oxidase, might function like a secondary respiratory chain that is active when oxygen or iron levels are too low for the cytoplasmic membrane-associated electron transport system to operate. This activity would allow cells expressing luciferase to reoxidize reduced coenzyme even when oxygen levels are low. Reoxidation of reduced coenzyme would permit cells of luminous bacteria in low oxygen habitats, such as in animal gut tracts, to continue to transport and metabolize growth substrates, gaining energy through substrate-level phosphorylation. Another possibility is that light production could facilitate dissemination of luminous bacteria. The feeding of animals on luminous particles (decaying tissues, fecal pellets, and moribund animals infected by luminous bacteria), to which they are attracted, would bring the bacteria into the animal's nutrient-rich gut tract for reproduction and dispersal. Other hypotheses for the function of luminescence in bacteria have been put forward, and future studies may provide support for one or more of these hypotheses. It is by no means clear yet what actual benefits, physiological or ecological, accrue to luminous bacteria that lead them to retain and express an energetically expensive enzyme system.

Evolution of the Bacterial Luminescence System

The natural presence of genes necessary for producing light defines the luminous bacteria. The necessary genes, *luxA* and *luxB*, encoding the luciferase subunits, *luxC, luxD* and *luxE*, for the fatty acid reductase subunits, and *luxG*, encoding a flavin reductase, are consistently found together as a cotranscribed unit, *luxCDABEG*. The reason for this conservation of *lux* genes as a unit is not known, but it might relate to efficient light production; the contiguous presence of these genes as an operon might help promote the coordinated production of luciferase and substrates for luciferase, long-chain aldehyde and reduced flavin mononucleotide ($FMNH_2$). The conservation of these genes as a unit in nearly all luminous bacteria examined (**Figure 7**) suggests that the *lux* operon arose in the distant past evolutionarily. Supporting this view, phylogenetic analysis demonstrates that the individual *lux* genes of different bacterial species are homologous, as was suggested by the high levels of amino acid sequence identities of the inferred Lux proteins. This homology implies that the bacterial *luxCDABEG* genes arose one time in the evolutionary past. The use by luciferase of oxygen as a substrate implies that this enzymatic activity originated after oxygenic photosynthesis by ancestors of modern-day cyanobacteria began to increase the level of O_2 on Earth, approximately 2.4 billion years ago, during the Great Oxidation Event. A marine origin for bacterial luminescence seems likely because most species of luminous bacteria are marine (**Table 1**).

Evolutionary Origin of Bacterial Luciferase

The origins of the individual genes of the *lux* operon remain obscure. Luciferase, however, might have arisen from a primitive flavoprotein, a flavin-dependent, aldehyde-oxidizing protoluciferase that incidentally produced a small amount of light. If the level of light produced by this protoluciferase was sufficient to be detected by phototactic multicellular animals, they might have been attracted to luminous particles containing these early bioluminescent bacteria and may have fed on them, introducing the bacteria into the animal's nutrient-rich digestive tract. This interaction might thereby have enhanced the reproduction of these bacteria and led to selection for more intense light output. The evolutionary steps leading to protoluciferase may also have involved selection for oxygen detoxification activity that permitted early protobioluminescent anaerobic bacteria to survive an increasingly aerobic environment before the evolution of cytochrome-dependent respiration. Bacterial protoluciferase, either a monomer or a homodimer, might have been encoded by a single gene, an early form of *luxA*. Modern-day α- and β-subunits of luciferase alone, however, are unable to produce high levels of light *in vitro* or *in vivo*. Alternatively, a light emitting bacterial protoluciferase might have arisen following gene duplication of the primitive *luxA* gene that is thought to have given rise to *luxB* (see below).

Most luminous bacteria are members of *Vibrionaceae*, which suggests that the bacterial luminescence system arose in the ancestor of this family. Recent identifications of new luminous species and of luminous strains of species not previously known to be luminous support this view. Assuming vertical inheritance of the *lux* genes from that ancestor, through its descendents to modern-day bacteria, gene phylogenies for luminous bacteria (**Figure 2**) imply a complex history of *lux* gene duplication, gene recruitment, and gene loss within this family. Phylogenetic analyses also suggest that the *lux* genes were acquired by the luminous species of *Shewanellaceae* and *Enterobacteriaceae* from a member or members of *Vibrionaceae*.

Gene Duplication

Based on amino acid sequence identities, a tandem duplication of the ancestral *luxA* gene, followed by sequence divergence, is thought to have given rise to *luxB*, leading to formation of the heterodimeric luciferase present in modern-day luminous bacteria. Similarly, a tandem duplication of *luxB* is thought to have given rise to *luxF*, which encodes a nonfluorescent flavoprotein; *luxF* is present in the *lux* operons, *luxCDABFEG*, of three of the four luminous *Photobacterium* species (**Figure 7**).

Gene Loss

Most species of *Vibrionaceae* lack the *lux* genes and therefore are nonluminous. Also, most strains of some luminous species, such as *V. cholerae*, are nonluminous. The low incidence of luminous species in the family suggests that the *lux* genes have been lost over evolutionary time from many of the lineages that have given rise to extant species. It also seems likely that nonluminous variants of luminous species can arise frequently through loss of the *lux* operon. The scattered incidence of *lux* genes in *Vibrionaceae* presumably relates to different ecologies of the different species. It is not clear, however, how having and expressing *lux* genes contributes to the life style of luminous bacteria, because there are no obvious ecological differences between luminous and nonluminous species except in the case of those species that are light-organ symbionts.

With respect to the loss of individual *lux* genes, *P. leiognathi* strains lack *luxF*, a gene that is present in the *lux* operons of other luminous *Photobacterium* species (**Figure 7**). Presumably, therefore, *luxF*, possibly acquired in the lineage leading to *Photobacterium* through duplication and sequence divergence of *luxB*, was lost from the lineage that gave rise to *P. leiognathi*. This loss might reflect the evolutionary divergence of *P. leiognathi* from other luminous species of *Photobacterium*. Also, some strains of *P. mandapamensis* bear nonsense mutations in *luxF*, further evidence that this gene does not play an essential role in the biology of *Photobacterium* species.

Mutations in *luxF* in *P. mandapamensis* might set the stage for loss of this gene from the *lux* operon. Similarly, the *lux* operons of *P. phosphoreum* strains lack *ribE*, one of the genes 'recruited' to the *lux* operon in *Photobacterium.*

Recruitment of Genes to the *lux* Operon

Linked to the luminescence genes in *Photobacterium* species, and apparently cotranscribed with them, are genes involved in synthesis of riboflavin, forming an operon of ten or 11 genes, *luxCDAB(F)EG-ribEBHA*, referred to as the *lux-rib* operon (**Figure** 7). The presence of the *ribEBHA* genes (just *ribBHA* in *P. phosphoreum*) appears to be a case of gene recruitment to the *lux* operon of *Photobacterium*, because these genes, with the exception in *V. harveyi* of *ribB* (referred to originally as *luxH*) are not contiguous with the *lux* operons of other species of luminous bacteria. Again, one can invoke the notion that the presence of genes for synthesis of riboflavin, a major component of FMN, as part of the *lux* operon might facilitate light production by ensuring coordinate synthesis of luciferase and of substrates for the enzyme. In this regard, it is interesting to note that in *A. fischeri, ribB*, which is not linked to the *lux* operon, is controlled by the LuxR/acyl-homoserine lactone-quorum sensing system that controls *lux* operon expression.

A second example of apparent gene recruitment to the *lux* operon is the presence of regulatory genes, *luxI* and *luxR*, which control *lux* operon transcription, in *A. fischeri* and *A. salmonicida.* In *A. fischeri*, the *luxI* gene is part of the *lux* operon, whereas *luxR* is upstream and divergently transcribed. In *A. salmonicida*, the arrangement is somewhat different, with two *luxR* genes flanking the *lux* operon and with *luxI* following the downstream *luxR* gene (**Figure 7**). In other species, the *lux* regulatory genes either have not been identified or, as in the case of a different (not homologous) *luxR* gene in *V. harveyi*, they are not linked to the *lux* operon. The grouping of regulatory genes with the *lux* operon in *Aliivibrio* might ensure a tight regulation of luminescence under quorum sensing control.

Horizontal Transfer of the *lux* Operon

In addition to gene duplication, loss, and recruitment, evidence is accumulating that *lux* genes have been acquired in some bacteria by horizontal gene transfer. Two species of *Shewanella* are luminous and carry *lux* operons similar to that of *A. fischeri*, suggesting acquisition from *A. fischeri* or an ancestor of this bacterium. Support for the notion of horizontal transfer of the *lux* operon from *A. fischeri* to luminous *Shewanella* species is seen in the presence of *luxR* and *luxI* genes in association with the *lux* operon of *S. hanedai* and in the same gene order as in *A. fischeri* (**Figure** 7). Recent evidence indicates that three *Vibrio* species, *Vibrio chagasii*, *Vibrio damselae*, and *Vibrio vulnificus*, acquired their *lux* gene by horizontal transfer. The situation for *Photorhabdus* is not yet clear, but an early transfer of *lux* genes from a member of *Vibrionaceae* might have occurred. These considerations suggest that the *lux* genes may have arisen within the lineage leading to modern-day members of *Vibrionaceae* and were then lost from several descendents, retained by some, and transferred relatively recently from a member or members of *Vibrionaceae* to *Photorhabdus* and *S. hanedai* and *S. woodyi.*

Natural Merodiploidy of the *lux-rib* Operon

An intriguing wrinkle in the evolutionary dynamics of the *lux* operon is that many strains of *P. leiognathi* isolated from nature carry two intact and apparently functional *luxCDABEG-ribEBHA* operons. This situation represents an unusual case of natural merodiploidy in bacteria, the presence of two or more copies of the same gene or genes in the genome of a bacterium, because of the large number of genes involved and because the second operon did not arise by tandem duplication of the first. The two *lux-rib* operons are distinct in sequence and chromosomal location. One operon is in the ancestral chromosomal location of the *lux-rib* operon in *P. leiognathi* and related bacteria. The other is located elsewhere on the chromosome and is present in many but not all strains of *P. leiognathi*; it is flanked by genes specifying transposases, which suggests it can transfer between strains. Phylogenetic analysis indicates that the two *lux-rib* operons are more closely related to each other than either is to the *lux* and *rib* genes of other bacterial species. This finding rules out interspecies horizontal transfer as the origin of the second *lux-rib* operon in *P. leiognathi*; instead, the second operon apparently arose in the distant past within, and was acquired by transposon-mediated transfer from, a lineage of *P. leiognathi* that either has not yet been sampled or has gone extinct. Merodiploidy of the *lux-rib* operon in *P. leiognathi* also is the first instance of merodiploid strains of a bacterium having a nonrandom geographic distribution; strains bearing a single *lux-rib* operon are found over a wide geographic range, whereas *lux-rib* merodiploid strains have been found only in coastal waters of Honshu, Japan (**Figure 8**). The presence of multiple copies of each of the *lux* and *rib* genes might provide opportunities for sequence divergence and selection that could lead to the evolution of new gene functions in one or the other of the duplicate genes.

Isolation, Storage, and Identification of Luminous Bacteria

When working with luminous bacteria, and particularly when isolating new strains from nature, the possibility that bacteria could be pathogenic should always be kept

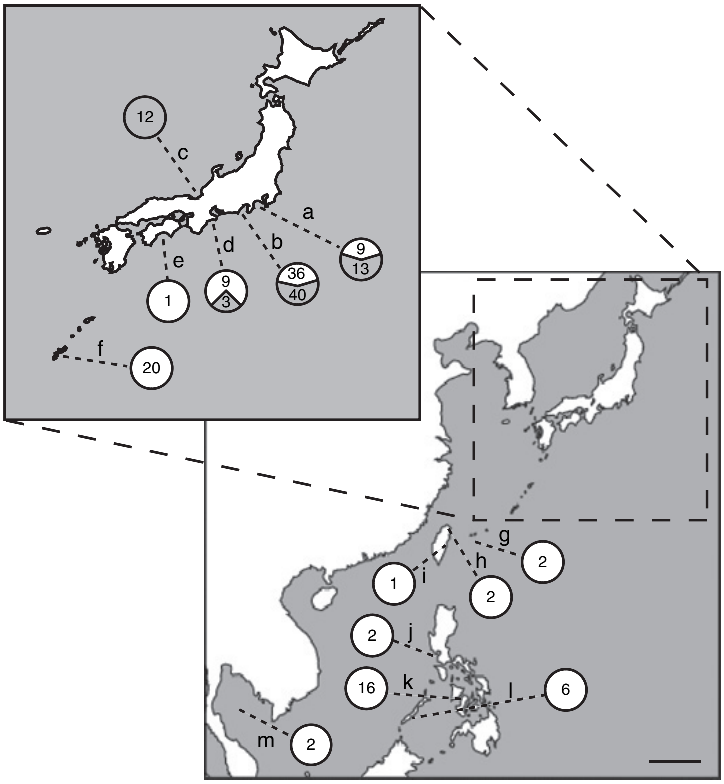

Figure 8 Nonrandom geographic distribution of *lux-rib* merodiploid strains of *P. leiognathi*. The numbers next to each location indicate the number of strains identified as bearing single (white area in circle) or multiple (gray area in circle) *lux-rib* operons. The insert shows an enlarged map of the main islands of Japan, with some landmasses omitted for clarity. The scale bar is approximately 500 km. Reproduced from Ast JC, Urbanczyk H, and Dunlap PV (2007) Natural merodiploidy of the *lux-rib* operon of *Photobacterium leiognathi* from coastal waters of Honshu, Japan. *Journal of Bacteriology* 189: 6148–6158.

in mind and appropriate care to avoid infection should always be used.

Luminous bacteria can be isolated from most marine environments, and two methods, direct plating of seawater and enrichment from marine fish skin, are effective and simple for this purpose. An easily prepared complete medium that is suitable for growing all known luminous bacteria contains: natural or artificial seawater, diluted to 70% of full strength, $10\,\mathrm{g\,l^{-1}}$ tryptone or peptone, and $5\,\mathrm{g\,l^{-1}}$ yeast extract, with $1.5\,\mathrm{g\,l^{-1}}$ agar for solid medium. Sugars and sugar alcohols (i.e., glycerol) are unnecessary and can lead to acid production and death of cultures; their use in isolation media should be avoided. For isolations from environments where high numbers of bacteria that form spreading colonies may be present, such as coastal seawater, sediment, and intestinal tracts of marine animals, the use of agar at 4% ($40\,\mathrm{g\,l^{-1}}$) is recommended. This harder, less moist agar limits the ability of bacteria motile on solid surfaces, for example, certain peritrichously flagellated bacteria and bacteria that move by gliding motility, to spread over the plate and cause cross contamination of colonies.

Directly plating of seawater involves simply spreading an appropriate volume, typically $10–100\,\mu\mathrm{l}$ for coastal seawater, of the sample on one or more plates and incubating at room temperature or, preferably, cooler temperatures, such as 15 °C to 20 °C. For open ocean seawater and other samples with a lower number of bacteria, larger volumes, for example, 100 ml to 1 l, can be filtered through membrane filters with a pore size of 0.2 μm or 0.45 μm to collect the bacteria. The filters are then placed, bacteria side up, on plates of the above medium. Once colonies have arisen, usually within 18 to 24 h at room temperature, the plates can be examined in a dark room. Luminous colonies can then be picked (sterile wooden toothpick are suitable for this purpose) and streaked for isolation on fresh plates of the same medium. Use of a red light, such as a photographic darkroom light, on a variable intensity control can make the picking of luminous colonies easier; by adjusting the red light, colonies of nonluminous bacteria can be made to appear reddish, whereas luminous colonies are blue due to their luminescence. Samples collected from warm waters and incubated at room temperature are more likely to yield *V. harveyi* and related luminous *Vibrio* species, as well as *A. fischeri, P. leiognathi* and *P. mandapamensis*; whereas cold seawater samples plated and incubated at lower temperatures are likely to yield *A. logei, P. kishitanii, P. phosphoreum*, and *S. hanedai*. It should be noted that some strains of *A. logei* and *S. hanedai* grow well but do not produce light at room temperature; attempts to isolate these and other psychrotrophic bacteria should be carried out at 15 °C.

Enrichment from fish (or squid) can be made using fresh samples and sterile seawater or frozen samples with natural, unsterilized seawater. The tissue, preferably with the skin on, is placed in a tray, skin up, covered halfway with seawater, incubated, and observed daily in the dark for luminous spots, which arise in one to a few days. These spots, colonies of luminous bacteria, can then be picked and streaked for isolation on the medium described above containing 4% agar. From fish and squid, a variety of different species of luminous bacteria can be isolated, especially when different incubation temperatures, such as 4 °C, 15 °C, and 22 °C, are used.

Storage at ultra low temperature, for example, from −75 °C to −80 °C, in a suitable cryoprotective medium is effective for all luminous bacteria. Cryopreservation of luminous bacteria is recommended to ensure their survival and to avoid the formation of dim and dark variants and the occurrence of other genetic changes. An effective cryoprotective medium for luminous bacteria is filter-sterilized deep freeze medium (2× DFM), prepared with 1% w/v yeast extract, 10% DMSO, 10% glycerol and 0.2M K_2HPO_4/NaH_2PO_4 (pH 7.0).

Phenotypic and genotypic traits were used in the past for identification of luminous bacteria and descriptions of new species. These methods remain useful, both for practical provisional identifications of new strains and as a complement to the construction of a DNA sequence-based molecular phylogenies for identification. With the advent of rapid, inexpensive DNA sequencing, the availability of many sets of primers for various genes whose sequences are useful in bacterial species resolution, and an expanded database of sequences for comparisons (e.g., GenBank), a DNA sequence-based approach to identification has become cost effective, rapid, and highly accurate. For luminous bacteria, phylogenetic analysis of *lux* and *rib* genes, together with housekeeping genes such as the16S rRNA gene, *gyrB*, and *pyrH*, for example, can quickly and accurately place a strain in a species or indicate the possibility that it may be new.

Complete characterization of a new species of luminous bacteria should include a multigene phylogenetic analysis together with examination of biochemical and morphological traits, DNA hybridization analysis, determination of the mol% G + C ratio, fatty acid profile analysis, and comparative genomic analysis such as amplified fragment length polymorphism (AFLP) or repetitive extragenic polymorphic PCR (rep-PCR). The examination of multiple independent isolates of the new entity and inclusion in the analysis of the type strains of all closely related species are critically important for accurate and definitive work. Increasingly, multigene phylogenetic analysis is becoming a standard for identification and characterization of luminous bacteria.

Conclusions

Knowledge of bioluminescent bacteria has increased greatly in recent years, through examination of their evolutionary relationships and symbioses and through the identification of new species and strains. Many new species of light-emitting bacteria will likely be identified in future studies, as new habitats are examined and as molecular phylogenetic criteria are used to discriminate among closely related species. The ability to distinguish among luminous bacteria using phylogenetic criteria opens up for detailed analysis questions of their habitat specificity, biogeography, and host specificity. Information gained from analysis of the *lux* operons of newly recognized luminous bacteria will likely provide further insight into the evolutionary dynamics of the *lux* operon and its horizontal transfer gene among members of *Vibrionaceae* and to members of other bacterial groups.

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Bioreactors

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Defining Statement
Introduction
Classifications of Bioreactors
Principles of Bioreactor Analysis and Design
Sensors, Instrumentation, and Control
Metabolic and Protein Engineering
Stability and Sterilization
Conclusions
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Glossary

airlift reactor Column with defined volumes for upflow and downflow of the culture broth; vertical circulation occurs because air is bubbled into the upflow volume.

batch bioreactor Culture broth is fed into the reactor at the start of the process; air may flow continuously.

bubble column reactor Aerated column without mechanical agitation.

fed batch Liquid media is fed to the reactor continuously; the broth accumulates in the reactor as there is no outflow of liquid.

heterotrophs Microorganisms growing on an organic compound that provides carbon and energy.

insect cell culture Cultivation of insect cells in a bioreactor to produce a protein or other product.

photoautotrophs Microorganisms that use light for energy and carbon dioxide for their carbon source.

plant cell culture Production of plant cells in a bioreactor to produce useful products.

protein engineering The design, development, and production of new protein products with properties of commercial value.

tissue engineering The design, development, and production of tissue cells (biomaterials) for use on or in humans.

Defining Statement

Bioreactors contain substrates, nutrients, and microbial cells in a controlled environment such that one or more useful products is produced. The goal of bioreactor design is to scale up from laboratory bioreactors to production scale vessels that produce the desired product economically and reliably.

Introduction

The importance of the bioreactor is recorded in early history. The Babylonians apparently made beer before 5000 BC. Wine was produced in wineskins, which were carefully selected for their ability to produce a beverage that met the approval of the King and other members of his sensory analysis taste panel. Food and beverage product quality depended on art and craftsmanship rather than on science and engineering during the early years of bioreactor selection and utilization. Early recorded history shows that some understood the importance of the reactants and the environmental or operating conditions of the reactor. This allowed leavened bread and cheese to be produced in Egypt more than 3000 years ago.

The process of cooking food to render it microbiologically safe for human consumption as well as to improve its sensory qualities is also an ancient tradition. The process of thermal inactivation of microorganisms through the canning of food to allow safe storage was an important early achievement in bioreactor design and operation.

As humans learned to live in cities, waste management including wastewater treatment emerged as a necessity for control of disease. One of the first process engineering achievements was the biological treatment of wastes in bioreactors designed and built by humans for that purpose. Because a significant fraction of the population of a city could die from disease spread by unsanitary conditions, these early bioreactors represented important advancements.

After microorganisms were discovered, microbiologists and engineers increased their understanding of the biochemical transformations in bioreactors. Simple anaerobic fermentations for the production of ethyl alcohol, acetone, and butanol were developed. Aerobic and anaerobic treatment of wastewater became widely used. Sanitary engineering became a part of civil engineering education.

In the 1940s, the field of biochemical engineering emerged because of developments in the pharmaceutical industry that required large-scale bioreactors for the production of streptomycin and penicillin. Progress in bioreactor design and control resulted from research on oxygen transfer, air and media sterilization, and pH control. The central concern of the early biochemical engineers was the development of bioreactors that could achieve and maintain the chemical and physical environment for the organism that the biochemist/microbiologist recommended. The ability to scale up from laboratory bioreactors to large fermentors required the development of instrumentation such as the sterilizable oxygen electrode. Early courses in biochemical engineering were concerned with the analysis, design, operation, and control of bioreactors. While the field of biochemical engineering is less than 60 years old and some of the pioneers are still available to provide a first-person account of those exciting days, great progress has been made in bioreactor engineering. Some of the significant developments in bioreactor technology are listed in **Table 1** together with the approximate date.

Table 1 Significant developments in bioreactor technology

Development	*Year*[a]
Fermented beverages	5000 BC
Pasteur's discovery of yeast	1857
First medium designed for culturing bacteria	1860
Trickling filter for wastewater	1868
Anaerobic digester	1881
Production of citric acid using mold	1923
Production of penicillin in a Petri dish	1928
Production of penicillin in small flasks	1942
Hixon and Gaden paper on oxygen transfer	1950
Air sterilization in fermentors	1950
Continuous media sterilization	1952
Aiba, Humphrey, and Millis biochemical engineering textbook on bioreactor design	1965
Continuous airlift reactor for production of yeast	1969
Advances in instrumentation and computer control	1970
Progress in airlift bioreactor design	1973
Recombinant DNA technology	1973
Insect cells grown in suspension culture	1975
Large-scale cell culture to produce interferon	1980
Insulin produced using bacteria	1982
Bioreactors for fragile cell cultures	1988
Textbook on plant cell biotechnology	1994
Textbook on protein engineering	1996
Textbook on tissue engineering	1997
Rapidly increasing number of bioreactors for renewable energy applications	2006

[a] The dates are approximate and indicative of periods of time when advances were moving from initial studies to published works or commercial use.

Brazil has had a growing ethanol production industry since about 1975. In 2004, ethanol production was about 4×10^9 gallons in Brazil, and equal to about 40% of fuel use in Brazil. Worldwide ethanol production was 2% of gasoline consumption in 2004. In 2006, there was rapid growth in the production of ethanol for use as a fuel in the United States. As the price of fuels from petroleum increases, anaerobic digestion and ethanol production processes become more competitive.

Classifications of Bioreactors

Several methods have been used to classify bioreactors. These include the feeding of media and gases and the withdrawal of products; the mode of operation may be batch, fed batch, or continuous. The classification may be based on the electron acceptor; the design may be for aerobic, anaerobic, or microaerobic conditions. In aerobic processes, the methods of providing oxygen have resulted in mechanically agitated bioreactors, airlift columns, bubble columns, and membrane reactors. The sterility requirements of pure culture processes with developed strains differ from those of environmental mixed-culture processes, which are based on natural selection. There are bioreactors in which the vessel is made by humans and natural bioreactors such as the microbial cell, the flowing river, and the field of native grass. In this article, the classification of bioreactors will be based on the physical form of the reactants and products.

Gas Phase Reactants or Products

Oxygen and carbon dioxide are the most common gas phase reactants and products. Others include hydrogen, hydrogen sulfide, carbon monoxide, and methane. Oxygen is a reactant in aerobic heterotrophic growth processes, whereas it is a product in photoautotrophic growth. Generally, the concentration of the reactants and products in the liquid phase in the microenvironment of the cell influences the kinetics of the cellular reaction. Mass transfer to and from the gas phase affects bioreactor performance in most processes with gas phase reactants or products. The anaerobic reactor is designed to exclude oxygen. In some cases, inert gases are bubbled into the anaerobic reactor to provide gas–liquid interfacial area to remove the product gases.

Because the solubility of oxygen in water is very low, the dissolved oxygen in the broth is rapidly depleted if oxygen transfer from the gas to the liquid phase is disrupted in aerobic processes. The distribution of dissolved oxygen throughout the reactor volume and the transient variation affect reactor performance. When mold pellets or biofilms are present, the diffusion of oxygen into the interior should be considered. A significant fraction of the bioreactor literature is devoted to oxygen transfer and the methods recommended for the design and operation of aerobic bioreactors. The phase equilibrium relationship is based on thermodynamic data, while the rate of oxygen transfer depends on the gas–liquid interfacial area and the concentration driving force. Mechanical agitation increases the gas–liquid interfacial area. Aeration provides the supply of oxygen, and it affects the gas–liquid interfacial area.

Oxygen has been supplied by permeation through membranes in cultures in which bubbles may damage shear-sensitive cells. The membrane area and concentration driving force determine the oxygen transfer rate in these bioreactors.

Most large-scale bioreactors have either oxygen or carbon dioxide among the reactants or products. In many anaerobic fermentations, the formation of carbon dioxide results in bubbling, and often no additional mixing is required for either mass transfer or suspension of the microbial cells. Methane is produced through anaerobic digestion of waste products. It is also a product of microbial action in landfills, bogs, and the stomach of the cow.

Packed bed bioreactors are used to biodegrade volatile organic compounds in air pollution control applications. The rhizosphere provides a natural environment where many volatile compounds in soil are transformed by microbial and plant enzymes.

Liquid Phase Reactants or Products

Many bioreactors have liquid phase reactants and products. Ethanol, acetone, butanol, and lactic acid are examples of liquid products that can be produced by fermentation. The kinetics of biochemical reactions depend on the liquid phase concentrations of the reactants and, in some cases, the products. The Monod kinetic model and the Michaelis–Menten kinetic model show that many biochemical reactions have first-order dependence on reactant (substrate) concentration at low concentrations and zero-order dependence at higher concentrations. Rates are directly proportional to concentration below $10\,\mathrm{mg\,l^{-1}}$ for many reactants under natural environmental conditions. At very high concentrations, inhibition may be observed.

Hydrocarbons that are relatively insoluble in the water phase, such as hexadecane, may also be reactants or substrates for biochemical reactions. Microbial growth on hydrocarbons has been observed to occur at the liquid–liquid interface as well as in the water phase. The oxygen requirements are greater when hydrocarbon substrates are used in place of carbohydrates. At one time, there was great interest in the production of microbial protein from petroleum hydrocarbons. The commercialization of the technology was most extensive in Russia and other Eastern European countries. The airlift bioreactor is uniquely suited for this four-phase process because of the tendency of the hydrocarbon phase to migrate to the top of the fermentor. The hydrocarbons are found suspended as drops in the water phase, adsorbed to cells, and at the gas–liquid interface. The cells are found adsorbed to hydrocarbon drops, suspended in the water phase, and at the gas–liquid interface. In the airlift fermentor, the vertical circulation mixes the hydrocarbons and cells that have migrated to the top of the fermentor with the broth that enters the downflow side of the column.

One of the oldest and most widely practiced fermentations is the microbial production of ethanol and alcoholic beverages such as beer and wine. Because ethanol inhibits the fermentation at high concentrations, the process of inhibition has been extensively studied for this fermentation. Ethanol affects the cell membrane and the activities of enzymes. This inhibition limits the concentration of ethanol that can be obtained in a fermentor. Because ethanol is also produced for use as a motor fuel, there is still considerable research on ethanol production. Because the cost of the substrate is a major expense, inexpensive raw materials such as wastes containing cellulose have been investigated.

Solid Phase Reactants or Products

There are many examples of bioreactors with solid phase reactants. The cow may be viewed as a mobile bioreactor system that converts solid substrates to methane, carbon dioxide, milk, and body protein. While the cow is a commercial success, many efforts to transform low cost cellulosic solid waste to commercial products in human-made bioreactors have not achieved the same level of success because of economics.

Solid substrates such as soybean meal are commonly fed into commercial fermentations. Through the action of enzymes in the fermentation broth, the biopolymers are hydrolyzed and more soluble reactants are obtained.

Many food fermentations involve the preservation of solid or semisolid foods such as in the conversion of cabbage to sauerkraut and meats to sausage products. Cereals, legumes, vegetables, tubers, fruits, meats, and fish products have been fermented. Some fermented milk processes result in solid products such as cheeses and yogurts.

Other examples include the composting of yard wastes, leaching of metals from ores, silage production, biodegradation of crop residues in soil, microbial action in landfills, and the remediation of contaminated soil.

In many of these fermentations, mixing is difficult or expensive. Transport of essential reactants may depend on diffusion; the concentrations of reactants and products vary with position. Rates may be limited by the transport of essential reactants to the microorganisms.

Most compounds that are present as solids in bioreactors are somewhat soluble in the water phase. For reactants that are relatively insoluble, biochemical reaction rates may be directly proportional to the available interfacial area. The surface of the solid may be the location of the biochemical transformation. An example of microorganisms growing on the surface of a solid substrate is mold on bread. To design bioreactors for solid substrates and solid products, the solubility and the transport processes as well as the kinetics of the process should be addressed.

Recently, there has been considerable progress in tissue engineering. The rational design of living tissues and the production of these tissues by living cells in bioreactors are advancing rapidly because of the progress in systems design and control for both *in vitro* flow reactors and *in vivo* maintenance of cell mass.

Microorganisms in Bioreactors

The rate of reaction in bioreactors is often directly proportional to the concentration of microbial biomass. In biological waste treatment, the influent concentration of the organic substrate (waste) is relatively low, and the quantity of microbial biomass that can be produced from the waste is limited. The economy of the operation and the rate of biodegradation are enhanced by retaining the biomass in the bioreactor. In the activated sludge process, this is done by allowing the biomass to floculate and settle; it is then recycled. The trickling filter retains biomass by allowing growth on the surfaces of the packing within the bioreactor.

A variety of immobilized cell reactors and immobilized enzyme reactors have been designed and operated because of the economy associated with reuse of cells and enzymes. In the anaerobic production of ethanol, lactic acid, and the other fermentation products, the product yield is greatest when the organisms are not growing and all of the substrate is being converted to products. Continuous processes can be designed in which most of the cells are retained and the limiting maximum product yield is approached. Ultrafiltration membrane bioreactors have been used to retain cells, enzymes, and insoluble substrates.

In nature, cells are retained when biofilms form along flow pathways. The biofilms allow microorganisms to grow and survive in environments where washout would be expected. The excellent quality of groundwater is the result of microbial biodegradation and purification under conditions where microbial survival is enhanced by biofilm formation and cell retention on soil and rock surfaces. The ability of microorganisms to survive even after their food supply appears to be depleted is well established; this accounts for our ability to find microorganisms almost everywhere in nature. When spills occur, organic substances will often be degraded by microorganisms, if the nutritional environment is balanced. Nitrogen, phosphorous, and other inorganic nutrients often must be added.

The concentration of cells adsorbed to the surface and the concentration in the water phase depends on an adsorption phase equilibrium relationship and the operating conditions. In many environmental applications, most of the cells are adsorbed to surfaces. However, in large-scale fermentors with high cell concentrations and rich media feeds, only a small fraction of the cells are found on surfaces.

Photobioreactors

Light is the energy source that drives photoautotrophic growth processes. Because light is absorbed by the growing culture, the intensity falls rapidly as the distance from the surface increases. Photobioreactors are designed to produce the quantity of product that is desired by selecting a surface area that is sufficient to obtain the needed light. Heat transfer is an important design aspect because any absorbed light energy that is not converted to chemical energy must be dissipated as heat.

Principles of Bioreactor Analysis and Design

The basic principles of bioreactor analysis and design are similar to those for chemical reactors; however, many biochemical processes have very complex biochemistry. The chemical balance equations or stoichiometry of the process must be known or investigated. The yield of microbial biomass and products depends on the genetics of the strain and the operating conditions. The consistency of data from experimental measurements can be evaluated using mass balances such as the carbon balance and the available electron balance.

Microorganisms obey the laws of chemical thermodynamics; some heat is produced in heterotrophic growth processes. The free energy change is negative for the complete system of biochemical reactions associated with heterotrophic growth and product formation. Thus, the chemical energy available for growth and product formation decreases as a result of microbial assimilation of the reactants.

The rate of growth and product formation depends on the number of microorganisms and the concentrations of the nutrients. The kinetics of growth and product formation are often written in terms of the concentration of one rate-limiting substrate; however, in some cases, more than one nutrient may be rate limiting. The kinetics must be known for rational design of the bioreactor.

Heat is evolved in microbial bioreactors. For aerobic processes, the quantity of heat generated (heat of fermentation) is directly proportional to the oxygen utilized. Thus, the heat transfer and oxygen transfer requirements are linked by the energy regularity of approximately 450 kJ of heat evolved per mole of oxygen utilized by the microorganisms.

Transport phenomenon is widely applied in bioreactor analysis and design. Many fermentation processes are designed to be transport limited. For example, the oxygen transfer rate may limit the rate of an aerobic process. Bioreactor design depends on the type of organism as well as the nutritional and environmental requirements. For example, in very viscous mycelial fermentations, mechanical agitation is often selected to provide the interfacial area for oxygen transfer. Likewise, animal cells that grow only on surfaces must be cultured in special bioreactors, which provide the necessary surface area and nutritional environment. In other cases, animals are selected as the bioreactors, because the desired biochemical transformations can best be achieved by competitively utilizing animals; cost and quality control are both important when food and pharmaceutical products are produced.

Sensors, Instrumentation, and Control

The ability to measure the physical and chemical environment in the fermentor is essential for control of the process. In the last 60 years, there has been significant progress in the development of sensors and computer control. Physical variables that can be measured include temperature, pressure, power input to mechanical agitators, rheological properties of the broth, gas and liquid flow rates, and interfacial tension. The chemical environment is characterized by means of electrodes for hydrogen ion concentration (pH), redox potential, carbon dioxide partial pressure, and oxygen partial pressure. Gas phase concentrations are measured with the mass spectrometer. Broth concentrations are measured with gas and liquid chromatography; mass spectrometers can be used as detectors with either gas or liquid chromatography. Enzyme thermistors have been developed to measure the concentration of a variety of specific biochemicals. Microbial mass is commonly measured with the spectrophotometer (optical density) and cell numbers through plate counts and direct microscopic observation. Instruments are available to measure components of cells such as reduced pyridine nucleotides and cell nitrogen. Online biomass measurements can be made using a flow cell and a laser by making multiangle light scattering measurements. Multivariate calibration methods and neural network technology allow the data to be processed rapidly and continuously such that a predicted biomass concentration can be obtained every few seconds.

The basic objective of bioreactor design is to create and maintain the environment needed to enable the cells to make the desired biochemical transformations. Advances in instrumentation and control allow this to be done reliably.

Metabolic and Protein Engineering

Genetic modification has allowed many products to be produced economically. With the use of recombinant DNA technology and metabolic engineering, improved cellular activities may be obtained through manipulation of enzymatic, regulatory, and transport functions of the microorganism. The cellular modifications of metabolic engineering are carried out in bioreactors. Successful manipulation requires an understanding of the genetics, biochemistry, and physiology of the cell. Knowledge of the biochemical pathways involved, their regulation, and their kinetics is essential.

Living systems are bioreactors. Through metabolic engineering, man can modify these living bioreactors and alter their performance. Metabolic engineering is a field of reaction engineering that utilizes the concepts that provide the foundation for reactor design including kinetics, thermodynamics, physical chemistry, process control, stability, catalysis, and transport phenomena. These concepts must be combined with an understanding of the biochemistry of the living system. Through metabolic engineering, improved versions of living bioreactors are designed and synthesized.

While many products are produced in microbial cells, other cell lines including insect cells, mammalian cells, and plant cells are utilized for selected applications. The science to support these various living bioreactors is growing rapidly and the number of different applications is increasing steadily. The choice of which organism to select for a specific product must be made carefully with consideration of biochemistry, biochemical engineering, safety, reliability, and cost. Both production and separation processes affect the cost of the product; however, the cost of product development, testing, regulatory approval, and marketing are substantial as well.

Proteins with specific functional properties are being designed, developed, and produced through applications of protein engineering. Through molecular modeling and computer simulation, proteins with specific properties are designed. Protein production may involve applications of recombinant DNA technology in host cell expression bioreactors. An alternative is to produce a protein with the desired amino acid sequence through direct chemical synthesis.

Stability and Sterilization

While beneficial genetic modification has led to many industrial successful products, contamination and genetic mutations during production operations have resulted in many batches of useless broth. Batch processes are common in bioreactors because of the need to maintain the desired genetic properties of a strain during storage and propagation. Continuous operation is selected for mixed culture processes such as wastewater treatment, where there is natural selection of effective organisms.

Bioreactors that are to operate with pure cultures or mixed cultures from selected strains must be free of contamination, that is, the reactor and associated instrumentation must be sterilizable. The vessels that are to be used for propagation of the inoculum for the large-scale vessel must be sterilizable as well. Methods to sterilize large vessels, instrumentation, and connecting pipes are well developed; however, there is a continuing need to implement a wide variety of good manufacturing practice principles to avoid contamination problems.

Steam sterilization has been widely applied to reduce the number of viable microorganisms in food and fermentation media. As temperature increases, the rates of biochemical reactions increase exponentially until the temperature affects the stability of the enzyme or the viability of the cell. The Arrhenius activation energies, which have been reported for enzymatic reactions and rates of cell growth, are mostly in the range of 20–80 $\mathrm{kJ\,g\,mol^{-1}}$, whereas activation energies for the thermal inactivation of microorganisms range from 200 to 400 $\mathrm{kJ\,g\,mol^{-1}}$. Many of the preceding principles also apply to the thermal inactivation of microorganisms in bioreactors. When solids are present in foods or fermentation media, heat transfer to the interior of the solid is by conduction. This must be considered in the design of the process because of the increase in the required sterilization time.

Conclusions

Bioreactors are widely used for a variety of purposes. The knowledge base for their application has increased significantly because of the advances in chemical, biochemical, and environmental engineering during the last 60 years. Many different bioreactors have been designed because of the importance of optimizing the production environment within each vessel for each application.

Many pharmaceutical, biomedical, biochemical, food, beverage, fuel, and biomaterial products are produced in bioreactors. The total amount and commercial value of these bioproducts increases annually.

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Caulobacter

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Defining Statement
Caulobacter and Caulobacters
Development in *Caulobacter crescentus*
Further Reading

Glossary

chemotaxis Motility directed by a sensory/motor system along a gradient of a solute.

cytoskeleton Protoplasmic proteins that participate in the development and/or maintenance of cell shape.

oligotrophy Of the habitat: Condition of the habitat characterized by low organic productivity and low density of microbial populations. Of microbes: Nutritional class of microbes whose competitiveness in their natural habitat depends on their ability to exploit a scarcity of nutrients.

periplasm Region immediately external to the cell membrane; in Gram-negative bacteria, the region is separated from the environment by a second superficial, outer membrane.

phosphorelay Systematic transfer of phosphoryl groups from a nucleotide triphosphate through a series of proteins, thereby altering the functional activity of each protein; important in many signal-transduction systems.

prostheca An outgrowth of the Gram-negative bacterial cell surface that includes the outer membrane, the cell membrane, and the peptidoglycan layer; may or may not include cytoplasmic elements.

sacculus The rigid peptidoglycan layer of a bacterial cell envelope; in Gram-negative bacteria, the sacculus lies within the periplasm.

stalked sibling The product of asymmetric fission in caulobacter that bears a prostheca and is not motile.

swarmer sibling The product of asymmetric fission in caulobacter that bears a flagellum and is motile.

TonB-dependent receptors Periplasmic proteins that assist in providing metabolic energy for active transport of solutes across the outer membrane of a Gram-negative bacterium into its periplasm.

transcription cascade System of sequential transcription of genes within which transcription of each 'later' gene requires activation by the product of an 'early' gene.

Abbreviation

GS/GOGAT	glutamine synthetase/glutamate-oxoglutarate amino transferase

Defining Statement

Caulobacters are aquatic, oligotrophic bacteria with a distinctive reproductive cycle that produces flagellated juvenile swarmers and nonmotile adolescent stalked cells as siblings. Correlations between their oligotrophy and their dimorphy are drawn against the background of description of the molecular details of development now available from extensive research with *Caulobacter crescentus.*

Caulobacter and Caulobacters

Prologue: It was not a dark and stormy night; it was an overcast day, and the sky was a smooth gray. Nevertheless, the water of the lake was blue; it was unquestionably clean and oligotrophic. A caulobacteriologist scooped samples from the lake's surface with great expectations. Within 2 weeks, the collector was rewarded by finding that 75% of the 3177 aerobic, chemoorganoheterotrophic bacteria per milliliter that formed visible colonies on a dilute peptone medium were caulobacters.

Recognizing Caulobacters

The genus Caulobacter

Species assigned to the genus *Caulobacter* are aquatic, oligotrophic, aerobic, freshwater, Gram-negative *Alphaproteobacteria.* All the distinguishing morphologic features of the genus *Caulobacter* are evident in the dividing cell (see **Figures 1–4**):

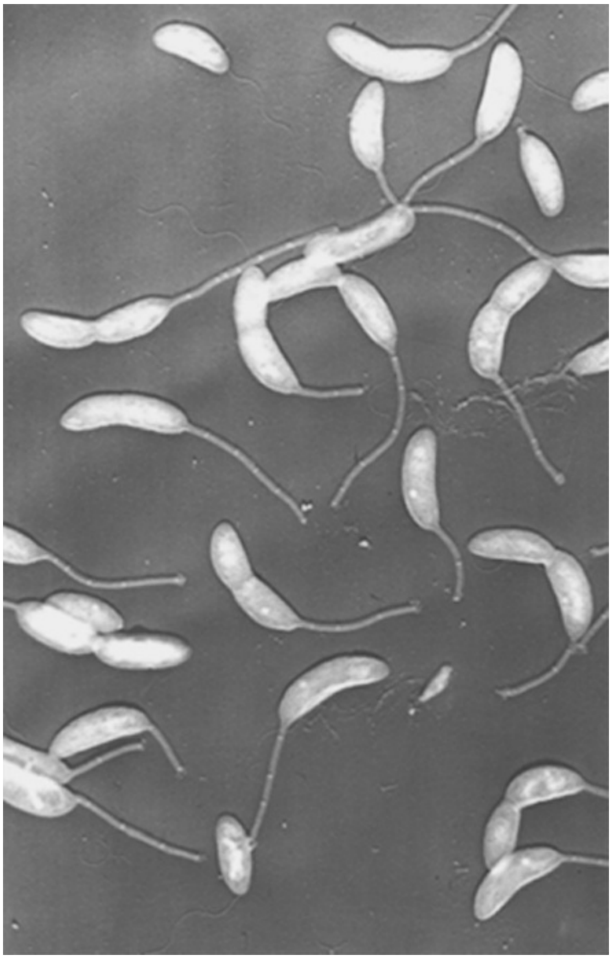

Figure 1 Vibrioid cells of *Caulobacter crescentus* strain CB2 grown in dilute peptone yeast extract medium. See also **Figure 8(a)**. EM, Pt-shadowed.

- An elongated, unicellular form – straight (rods or fusiform cells), curved (curved rods or subvibrios), or twisted (vibrios) – with poles that are blunt (rods) or tapered (vibrios, subvibrios, and fusiform cells);
- A different appendage at each pole: a single flagellum at the younger pole, and at the older pole a prostheca composed of the cell envelope (outer membrane, peptidoglycan layer, and inner membrane);
- A discrete blob of adhesive material, the 'holdfast', at two sites: at the flagellated pole at the base of the flagellum, and at the outer tip of the prostheca (hence its designation as a cellular 'stalk');
- A constriction at approximately the midcell that, when completed, will separate a flagellated 'swarmer' cell from its nonmotile stalked sibling; and
- Ringed disks of peptidoglycan and proteins, called stalk bands, at intervals along and within the stalk; one band is installed during each cell cycle and records the number of fissions completed by the cell since it began to develop its stalk. The rings look very much like the flagellar base plates first described in spirilla.

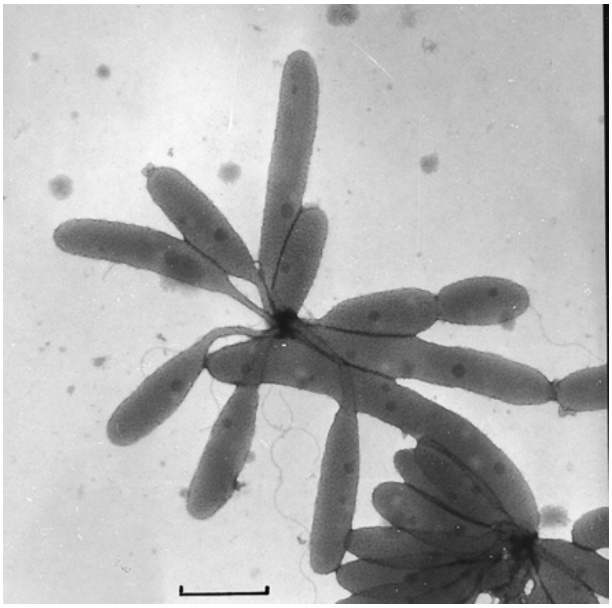

Figure 2 Bacteroid cells of *Caulobacter* sp. strain CB417 grown in dilute peptone yeast extract medium. EM, negatively stained with $AmMoO_4$. Marker = 1 μm.

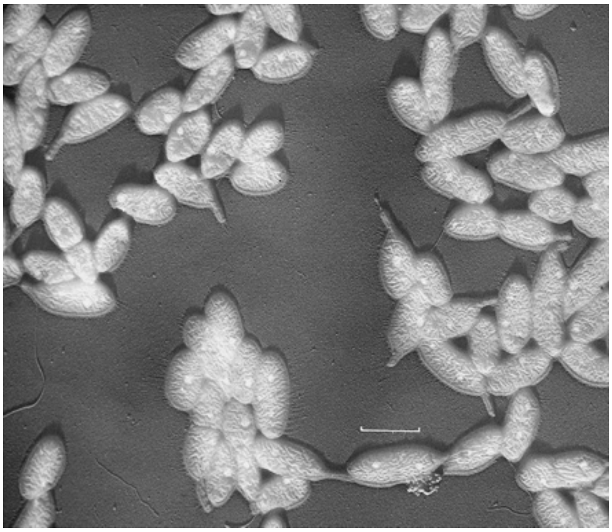

Figure 3 Fusiform cells of *Caulobacter leidyi* strain CB37 grown in dilute peptone yeast extract medium. See also **Figure 8(b)**. EM, Pt-shadowed. Marker = 1 μm.

Other caulobacters

Variations on this morphology are exhibited by closely related *Alphaproteobacteria.* The closest relative is the genus *Asticcacaulis* (literally, nonadhesive stalk). Although not adhesive, *Asticcacaulis* stalks are banded. The genus is distinguished from *Caulobacter* by several morphologic features.

- Cells of all isolates are blunt rods.
- Locations of the holdfast, flagellum, and prostheca are not coincidental; the holdfast is located excentrically

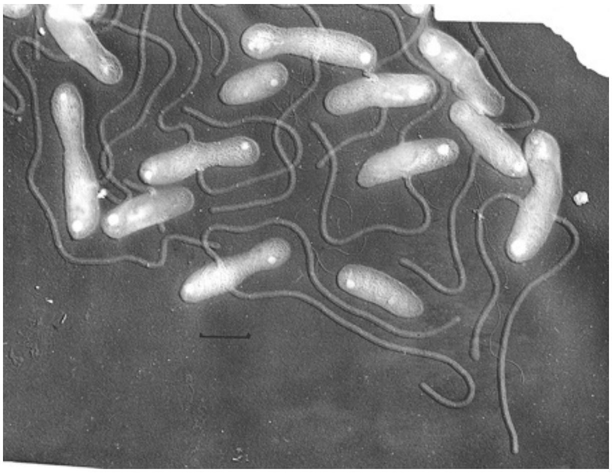

Figure 4 Biprosthecate cells of *Asticcacaulis biprosthecum* strain C-19 grown in dilute peptone yeast extract medium. EM, Pt-shadowed. Marker = 1 μm.

on the pole, the flagellum excentrically at the same pole, and the prostheca may be excentral, subpolar, or lateral. Isolates with lateral prosthecae may bear one or two prosthecae, although only one flagellum.

- Fission occurs by septation rather than constriction.
- A difference in size of the siblings, with the swarmer regularly much shorter than its stalked sibling during and immediately after fission, and a greater difference in cell cycle times between the siblings (see **Figure 9**).

Both *Caulobacter* and *Asticcacaulis* fall into the 16S rRNA family 'Caulobacteraceae', as do *Brevundimonas* (a composite taxon of phenotypically diverse bacteria) and *Phenylobacterium* (a genus of nonprosthecate bacteria). Some of the species currently assigned to *Brevundimonas* were originally named as *Caulobacter* species because they possess all the ecologic, morphologic, and physiologic features described for that genus.

Two other 16S rRNA families of *Alphaproteobacteria* include genera with caulobacterial morphologies. One species (*Caulobacter leidyi*) is placed in the family Sphingomonadaceae on the basis of its lipid content as well as its 16S rRNA. Isolates similar to *C. leidyi* are known, but not yet named or classified. The family Rhodobacteraceae includes the marine caulobacters of the genus *Maricaulis*. Dividing cells of these *Alphaproteobacteria* are identical to *Caulobacter* in microscopical appearance of their dividing cells, and their stalks are adhesive; however, stalk bands are typically absent.

The Caulobacterial Stalk

The term 'prostheca' was introduced by J.T. Staley to designate an outgrowth of the bacterial cell surface that includes at least the peptidoglycan sacculus (**Figure 5**); this definition distinguishes prosthecae from proteinaceous structures such as flagella and pili/fimbriae, and from extracellular polysaccharidic capsules and slime stalks. Known prosthecate bacteria are Gram negative, but not all are *Alphaproteobacteria*. Among dimorphic prosthecate bacteria that produce swarmer cells, the features of the dividing cell described above for caulobacters are – as far as known – unique to the three families of *Alphaproteobacteria* already mentioned. The caulobacterial

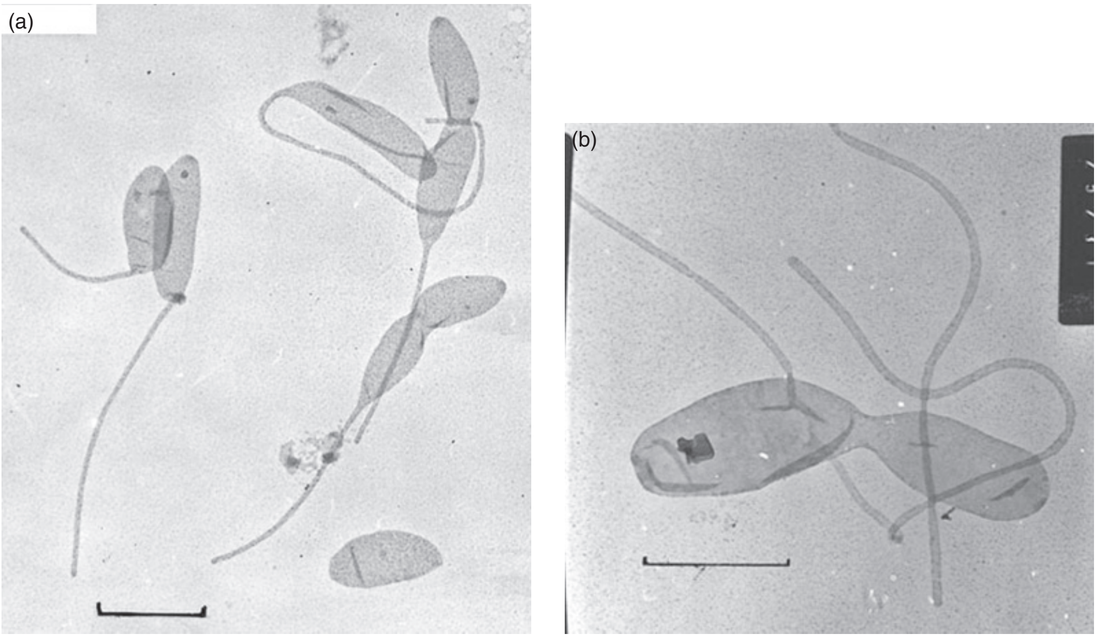

Figure 5 Peptidoglycan sacculi of caulobacters. EM, positively stained with uranyl and rotary shadowed with Pt. Marker = 1 μm. (a) *Caulobacter crescentus* strain CB2. (b) *Asticcacaulis biprosthecum* strain C-19. Marker = 1 μm.

prostheca lacks cytoplasm and DNA. It does not generate progeny, a reproductive process that occurs in the dimorphic bacteria of the genera *Hyphomicrobium* and *Hyphomonas*, where the nonadhesive prostheca produces swarmers by budding from its distal tip.

The value of motility in an aquatic organism is never debated. Adhesion, likewise, is generally regarded as adaptive because it can anchor the organism in a favorable locale. Although adhesion of a bacterium typically does not require a prostheca, adhering bacteria are very often perpendicular to the substratum. While this allows most of the cell surface to continue in contact with the liquid phase of the aquatic environment, an adhesive prostheca increases that advantage by preventing crowding of the cell body against other bacteria attached to the same substratum.

Only among the caulobacters is the prostheca typically adhesive, and adhesiveness is not a universal property even within this group. It is therefore an unavoidable challenge to infer possible functions for the stalk other than a role in adhesion. To date, three further functions have been proposed: (1) increased resistance to settling in an aquatic environment ('buoyancy'); (2) increase of the nutrient uptake-mediating area of the cell surface; and (3) boosting the incipient swarmer above the crowd to free it from entrapment in a biofilm. (1) The difference in buoyancy of nonstalked and stalked siblings is the mechanical basis for segregation of swarmers from their stalked siblings by differential centrifugation, which is the most widely used method for the establishment of synchronously developing populations used in cell cycle research with caulobacters (see section 'Cytoskeleton'). (2) Besides straightforward geometric reasoning, there is biochemical, physiological, developmental, and molecular (genetic and proteomic) evidence that supports the proposed role of the stalk in nutrient scavenging. Even stalks severed from the cell body mechanically or as a consequence of genetic mutation are demonstrably capable of accumulating solutes, and to consume metabolic energy doing so. (3) Observations of caulobacters attached to natural materials reveal only dispersed and not crowded caulobacters; swarmers tend to be well away from their stalked siblings before settling down. However, on both animate and inanimate surfaces exposed to caulobacter populations at 1000-fold or greater than their usual density in natural waters, and in the absence of predators such as protozoa and microfauna, caulobacters can be forced into crowds – a technique that can be useful in technological applications that employ immobilized caulobacters.

Accordingly, the caulobacter stalk is demonstrably capable of providing at least four functions. It mediates adhesion in a fashion that simultaneously exposes most of the cell surface to the liquid surroundings, the source of the cell's nutrients and lifts the cell body above a biofilm, promoting free dispersal of the motile sibling. Because the stalk engages in little biosynthetic activity except at the cell-stalk junction (see section 'Placement of cytoskeletal proteins and cell wall synthesis complexes'), it could serve (like microvilli in a mammalian intestine) to increase the ratio of nutrient-absorptive surface to nutrient-consuming protoplasm. Finally, it provides the capacity for the third ecologic habitat available in an aquatic environment: floating, as well as swimming in the water column and settling on submerged surfaces. Stalked caulobacters not attached to submerged surfaces accumulate in the neuston, or air–water interface, the shallow vertical zone in an aquatic habitat where organic substances accumulate because they diffuse less freely there than in the water column; where O_2 enters from the atmosphere; and where the local phototrophs, the primary producers, tend to gather during the day and catch the sun's energy that will support the local chemoheterotrophs such as caulobacters. Thus, bacteria with this peculiar morphology and developmental cycle are well suited to living competitively in low-nutrient-flux aquatic environments as aerobic, metabolically efficient, nutrient-scavenging chemoorganoheterotrophs. As mentioned in the Prologue, it is precisely where they are most dependably found as the predominant (but not crowded) aerobic, nonphotosynthetic bacteria.

Capture and Cultivation of Caulobacters

The natural environment in which caulobacters thrive and can sometimes even predominate is low-nutrient-flux water, both fresh and marine. Even when predominant, caulobacters do not accumulate as dense populations; they are rarely as crowded as 10^5–10^6 cells per milliliter. Natural waters are almost never constant in nutrient flux; even the most oligotrophic water will experience transient surges of nutrients that will support a responding increase in the density of the microbial populations and of the organisms that feed on the microbes. During such periods, caulobacters do not necessarily disappear, but they do not respond by multiplying rapidly. Consequently, they can be overwhelmed numerically by faster-growing bacteria such as pseudomonads and become difficult to detect – microscopically or by cultivation.

The most dependable way to encourage caulobacters to accumulate in a culture and to enable their isolation as colonies on solidified media is to depend on their development as a dimorphic population. Let the water sample stand in a tall bottle (such as a milk dilution bottle) at room temperature in the dark. In 4–14 days, a thin, delicate surface film of caulobacters will accumulate as the swarmers rise toward the air–water interface and then develop their 'water wings' – their stalks. Their accumulation can be accelerated by adding peptone to no more 0.01% (w/v); higher concentrations will encourage the multiplication of bacteria that can grow faster, but are less capable of scavenging nutrients from low concentrations. Similarly, incubation in the dark reduces primary production by phototrophs in the sample. Their activities interfere with the enrichment of caulobacters in two ways: by producing and exuding organic compounds, often amino acids, thereby enriching the water; and by serving as attachment surfaces for the caulobacters and reducing their buoyancy. The stalked bacteria probably do quite well attached to phototrophs, but they are harder to find because they sink out of the surface film.

A sample of surface film can be examined in a wet mount to determine whether the relative abundance of stalked cells is increasing, and when it is, a sample can be streaked on an unbuffered medium containing 0.05% peptone and not more than 1% agar, and incubated at room temperature. As colonies begin to appear, it is instructive to mark those that are macroscopic by the third day of incubation; in the author's experience, such colonies are never generated by caulobacters from enrichment samples. After 1–2 weeks, new colonies no longer appear and the preparation for screening can begin.

There is a fast, frustrating way to screen, and a tedious, fruitful way. The fast way is to examine samples of colonies in wet mounts. Because the medium is dilute, the colonies are very small (rarely more than 0.5–0.8 mm), and a colony (especially if cohesive) is easily consumed in the preparation of the mount; consequently, finding them is not capturing them. The tedious way is to patch a sample of each colony onto a gridded plate of medium, incubate for about 2 days, and then prepare wet mounts from the growth in the patches. This actually saves considerable time because the patches can be sampled in a pattern and marked off if they contain caulobacters, whereas using primary colonies requires that the colonies be mapped. Better yet, the patch is not consumed in the preparation of a wet mount.

Water can be surveyed for the presence of caulobacters by molecular tools such as FISH probing. There are, however, practical problems with fluorescence microscopical techniques. For example, natural populations of caulobacters are sparse, whether they are predominant or vastly outnumbered by noncaulobacters. It is often more difficult to detect them as fluorescent dots than as bacteria that wave about on their stalks in a focal plane above other bacteria settled on the slide. Their motion reveals their presence and their stalks identify them. Fluorescence intensity of a probe-binding caulobacter cell may be low and difficult to discern, for more than one reason: (1) Caulobacter cells in wild samples are relatively small, often not much wider than 0.5 μm, binding too little probe to be discerned readily; (2) Ribosomal content is often very low in cells from oligotrophic conditions, and relatively little probe is bound; (3) Most of the 'universal' probes used in community surveys are intended to bind to 16S rRNA. However, the caulobacters most frequently encountered in freshwater are members of the rRNA family Caulobacteraceae, the only family in the order Caulobacterales, which is a fairly discrete and congruous taxon as currently defined. Again, reaction with a fluorescent probe may be weak, and even competent application of probes can fail to detect a caulobacter signal in a sample that will yield a dozen or more different kinds of isolates – from which more can be inferred than just natural distribution.

To identify the colonies or patches containing stalked bacteria, the wet mount should be examined with phase-contrast illumination; the hydrated stalk is about 0.2 μm in diameter, just at the limit of resolution of a good phase-contrast microscope. If only ordinary light is available, preparing the mount in a droplet of dilute methylene blue will provide some contrast and help reveal some stalks. When crowded (as when growing on an agar surface), caulobacters adhere to each other in rosettes (see **Figures** 6 and 7), and as their stalks elongate they will appear in one or another arrangement. In *Caulobacter*-like organisms, the stalks will be oriented like the spokes of a wheel, with the cell bodies sticking outward toward the rim of the wheel or like pins in a pincushion (**Figure 6**). In *Asticcacaulis*-like organisms, the cells will appear to be in contact with each other at the poles, with the nonadhesive prosthecae extending from the cell cluster. *Asticcacaulis biprosthecum* rosettes (**Figure 7**) are especially distinctive as 'hairy' rosettes. The remainder of the procedure is a routine bacteriological restreaking until only one type of colony can be found on plates streaked from successive colony samples.

Colonies of caulobacters may be translucent or opaque. They occur in various shades of yellow/gold/

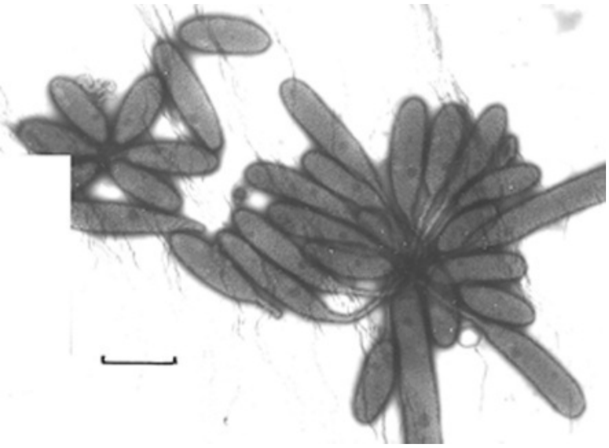

Figure 6 Newer and older rosettes of *Caulobacter* sp. strain CB417. The newer rosette includes six cells with almost no stalk elongation yet; the older rosette includes 21 cells, most of which have developed stalks. EM, negatively stained with $AmMoO_4$. Marker = 1 μm.

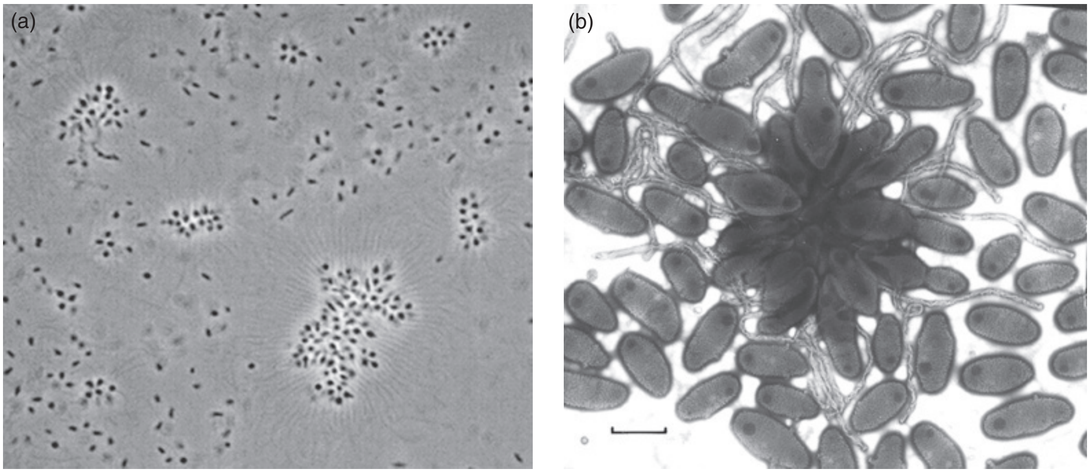

Figure 7 Rosettes of *Asticcacaulis biprosthecum* strain AC402 appearing 'hairy' due to the extension of the nonadhesive prosthecae away from the point of mutual adhesion of the cells. (a) Phase-contrast photomicrograph. (b) EM, negatively stained with $AmMoO_4$. Marker = 1 μm.

orange/red-orange due to cell-associated carotenoid pigments; pale pink due to an unidentified nonheme, noncarotenoid pigment; or colorless (unpigmented). As they age, colorless colonies often become dark tan-pink or pink-red due to heme accumulation. The colony margins are entire, the outline is circular, and the elevation is convex. Texture may be soft and watery, butyrous, compact and slightly sticky, or very cohesive and smooth or granular. Under each strain's optimal conditions of cultivation, colonies of most isolates become visible macroscopically between 60 h and 4 days of incubation – 3–4 days being most common. In liquid culture, doubling times of recent isolates range from 2.5 to 5–6 h, which is somewhat faster than they seem to manage in nature. As determined by the rate of accumulation of bands in the stalks of caulobacters captured by their adhesion to EM grids submerged in a mesotrophic lake, the reproductive rate *in situ* in early summer is three generations per day or 8 h per generation.

Faster-growing clones with doubling times close to only 2 h have been generated by laboratory cultivation from at least two species (*Caulobacter crescentus* and *C. leidyi*). These two species are the only caulobacters that can be grown in chemically defined media; vitamins (riboflavin, biotin, or cyanocobalamin) are required by some isolates, but are not sufficient for growth without complex supplementation with low concentrations of peptone, casamino acids, or yeast extract. Amino acids – commonly exuded by algae – are generally their preferred sources of both carbon and nitrogen; besides amino acids, many organic acids and several sugars can be consumed as carbon sources. Cellobiose, the disaccharide produced by the digestion of cellulose, is used by almost all isolates in the author's collection; this might explain in part the frequent occurrence of cytophagas in successful caulobacter enrichments.

Morphogenesis and Oligotrophy

It has long been suspected that one of the most important functions of the stalk is to increase the capacity of the caulobacter cell to scavenge nutrients from the oligotrophic environments where caulobacters are competitive. Very early in the studies with pure cultures of caulobacters, it was discovered that the nutrient with the most dramatic influence on morphogenesis of the stalk was phosphate: phosphate inhibits stalk elongation, and phosphate limitation results in a marked acceleration of stalk outgrowth (see **Figure 8** and section 'Phosphate and stalk elongation'). These observations were a stimulus to the notion that stalk development improves specific nutrient scavenging by the caulobacter cell, at least for phosphate. The inhibitory effect of phosphate can be mitigated by calcium, and the accelerating effect of phosphate scarcity requires available calcium. Although an abundance of the molecular details of development has been elucidated in *C. crescentus*, the interaction of phosphate and calcium in stalk elongation is yet to be explained in comparable detail. Nevertheless, in the natural habitat of caulobacters, phosphate and calcium are not typically available in excess together because of the insolubility of calcium phosphate; in caulobacters, calcium could serve as an indicator of phosphate scarcity.

Nitrogen-source availability has little demonstrable effect on stalk elongation, but starvation or limitation for nitrogen has a marked effect on stalk initiation (see section 'Nitrogen source and stalk initiation'). In nitrogen-adequate media in the laboratory, the swarmer cell sheds its flagellum and begins its maturation (initiating stalk development and DNA synthesis) after a motile stage that lasts about 25–33% of the cell cycle time. In nitrogen-inadequate media, the transition from motile, juvenile swarmer to nonmotile, reproductively capable stalked cell is postponed indefinitely and may not occur

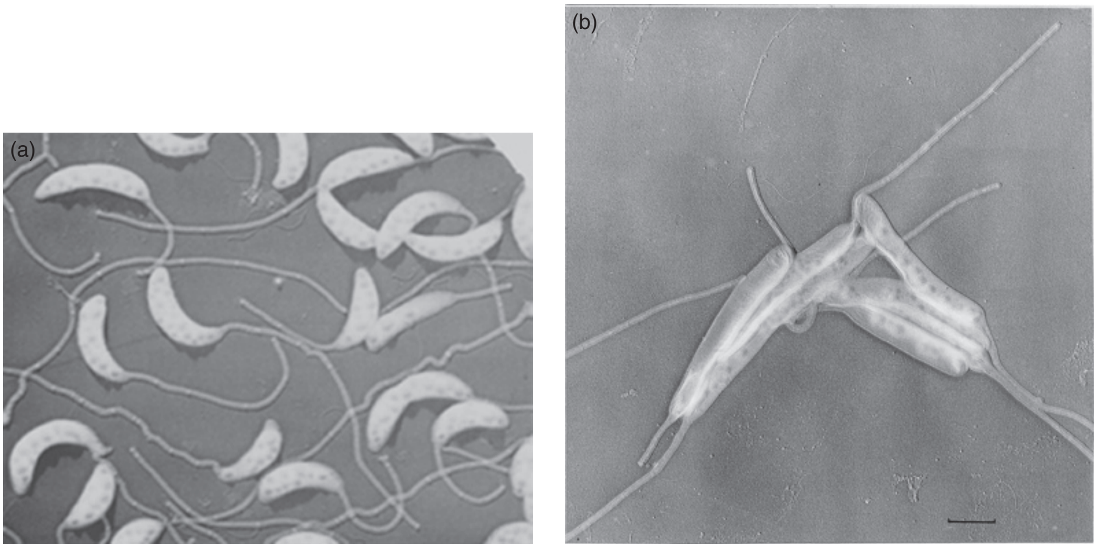

Figure 8 Cell and stalk elongation in phosphate-exhausted cultures of caulobacters. EM, Pt-shadowed. Marker = 1 μm. (a) *Caulobacter crescentus* strain CB2; See also **Figure 1**. (b) *Caulobacter leidyi* strain CB37; see also **Figure 3**.

until nitrogen becomes available. The only nutrient-specific chemotactic response so far demonstrated in *C. crescentus* by the Adler capillary assay is the attraction of nitrogen-limited swarmers toward nitrogen sources (ammonium and amino acids); in contrast, even swarmers produced under nutrient-limited conditions (whether for carbon or nitrogen or phosphorus) do not clearly respond to carbon sources or phosphate. Thus, two types of observation reveal that nitrogen limitation is the only nutritional signal that results in perpetuation of motility, in specific chemotaxis, and in delay of maturation of the swarmer into the reproductive stage. Together, these observations imply that the motility of the swarmer stage serves within the cell cycle to lead the cell toward a nitrogen-adequate site.

Caulobacters generally prefer amino acids as their nitrogen source, but they can consume inorganic nitrogen. However, *C. crescentus*, at least, lacks glutamic dehydrogenase and depends on the high-affinity GS/GOGAT (glutamine synthetase/glutamate-oxoglutarate amino transferase) pathway for the assimilation of ammonium. As in enteric bacteria, the activity of the *C. crescentus* system is inhibited and its synthesis is repressed by ammonium. As a consequence, *C. crescentus* growth is ammonium sensitive in minimal media, and the bacteria can starve for nitrogen in the midst of abundance when ammonium is the only nitrogen source available. In laboratory cultures, relief of that interference is achieved by the supplementation of defined media with glutamate to enable ammonium assimilation via GS/GOGAT or by the substitution of nitrate for ammonium.

Limitation for any one of the three macronutrients carbon, nitrogen, and phosphorus dramatically increases the uptake rate and affinity specifically for the limiting nutrient. Again, as in the case of stalk hypertrophy, phosphate limitation has the most striking effect: the relative increase in uptake rate (calculated as per unit area of cell surface) is about 80-fold for phosphate, but only about eightfold for carbon or nitrogen sources. Uptake of nonlimiting nutrients does not change with the stalk length. The long-stalked cells of phosphate-limited populations take up phosphate rapidly and with high affinity through both the cell body and the stalk, and their swarmer siblings exhibit a comparable enhancement of phosphate uptake. Nevertheless, while the stalk surface seems no more active in nutrient uptake than the rest of the cell, it lacks cytoplasmic components such as ribosomes and DNA. Thus, the hypertrophied stalk should provide the cell with an extension of the supply route for nutrients without increasing the nutrient demand by the most expensive metabolic activity, which is protein synthesis, or by the demand for phosphorus by nucleic acid synthesis.

A further aspect of caulobacterial nutrient-scavenging capacity was revealed in the fully sequenced genome of *C. crescentus*. There are in that genome loci that are interpreted as genes for TonB-dependent receptors, which are components of the systems for transducing metabolic energy to the outer membrane of Gram-negative bacteria that enables active uptake of solutes into the periplasm. Enteric bacteria have a few (not more than ten) such genes, and there are 34 in the highly versatile and competitive bacterium *Pseudomonas aeruginosa*. *C. crescentus* possesses 65. At least 19 such receptors appear in the proteome of isolated stalks, providing the strongest molecular evidence that the stalk is capable of energized, high-affinity uptake of solutes. Translocation to the cell body cytoplasm of solutes taken into the periplasm of the stalk probably involves periplasmic ATP-binding proteins, but the mechanism of translocation is still hypothetical.

In the oligotrophic waters that are the natural habitat of caulobacters, primary production of organic carbon is typically limited by the availability of phosphate or – less often – of nitrogen compounds, particularly nitrate. The dimorphy of caulobacters presents itself as a composite of adaptations that fits their ecologic role as nutrient scavengers, with the motile stage sensing nitrogen adequacy for stalk development and stalk elongation proceeding as needed for phosphate acquisition. The remaining major nutrient is organic carbon, which they presumably take up and transport into the cytoplasm with their exceptional battery of high-affinity TonB-dependent receptors. Those systems may enable caulobacters to collect the by-products of photosynthetic CO_2 fixation as those substances are released by the phototrophs to which caulobacters so commonly adhere and with whom they must compete for phosphorus and nitrogen.

Nutrient gradient-directed motility, extension of nutrient uptake surface relative to cytoplasm, nutrient uptake assisted by high-affinity TonB-receptor systems, and adhesion to phototrophic microbes all combine to make typical caulobacters highly competent for oligotrophy. Their unique reproductive cycle, which produces one offspring that will move away from its immobile sibling, also largely ensures that siblings will not crowd each other and therefore will not compete for sparse nutrients. Some of the details of the mechanisms that confer on caulobacters' unique approach to the problem of resource acquisition are described in the next section.

Development in *Caulobacter crescentus*

In 1935, on the basis of microscopical observations of mixed, wild (uncultivated) populations of caulobacters, Henrici and Johnson correctly inferred that a swarmer cell would develop a stalk and then proceed to asymmetric fission of a cell with a flagellum at one pole and a stalk at the opposite pole. They observed that such fission generated two siblings: a flagellated swarmer cell and a nonmotile sibling bearing a stalk. However, because they lacked pure cultures, they could not determine whether a swarmer cell could divide without first attaching to a substratum and/or developing a stalk. This double question was answered in the first extensive studies with pure cultures of a variety of caulobacters, during which it was found that mechanical segregation of nonstalked (swarmer) cells from stalked cells allowed separate cultivation of each cell type. Early studies with such populations revealed that newborn swarmer cell populations were synchronous with respect to flagellum shedding, stalk development, and completion of fission, and that fission was invariably preceded by the other two events. Synchrony in a stalked cell population was less stringent; nevertheless, a full round of cell division was completed in about 75% of the time required for a full round of cell division in a population that began as swarmers.

Continuous direct observation of mixed populations in microcultures revealed behavior that was entirely consistent with the behavior of the synchronous cultures: the cell cycle was significantly shorter for a cell that entered the cycle with its stalk than for a cell that was observed from its motile stage, and the latter type of cell became nonmotile and began to develop a stalk before it elongated, constricted, and divided. Fission of nonstalked cells was not detected. The absence of any difference in sequence or timing of morphogenetic and cell cycle events in populations attached to glass cover slips or suspended in agitated liquid cultures implied that adhesion was not required as a signal for flagellum shedding or the initiation of stalk outgrowth.

Within a few years after these initial studies, one species, *C. crescentus*, attracted the attention of microbial cell biologists who began to explore its dimorphic cell cycle. In the hands and minds of these diligent scientists, *C. crescentus* has proved a fertile experimental system that has yielded details of the mechanisms that enable a unicellular bacterium to order and integrate its morphogenesis, reproduction, and physiologic properties. The studies have also established that this 'simple' organism is a complex cell in which functional compartments can be established by the localization of proteins, without the elaboration of intracellular barrier membranes so familiar in eukaryotic cells.

Although there are some variations (rigidly enforced within a species), the cyclical sequence of developmental events in the lives of all caulobacter cells is largely a story of poles. The entire tale can be perceived at every *C. crescentus* pole from the birth of the pole as a product of fission to its maturation as an adhesive, banded stalk. It begins with the formation of the pole when fission is completed in generation I (see **Figure 9**). During generation II, begun by that fission, the sequence of developmental events at each new pole will be the same, but the time course of those events will depend on the age of the opposite pole; developmental events will occur sooner at the new pole of the sibling with a stalk at its older pole (in the 'stalked sequence' in **Figure 9**) than at the flagellated cell's new pole (in the 'swarmer sequence' in **Figure 9**). Maturation of each pole formed in generation I will occur in generation III. In short, the pole's story has three chapters:

1. The pole is formed by cell fission.
2. Extrusion events occur that confer motility, adhesiveness, and phage reception.
3. The flagellum and motility and phage receptors are lost and the pole elongates as a stalk.

The long version of this story is now remarkably detailed as the result of research that has employed mutations, inhibitors (particularly, inhibitors of cell wall synthesis),

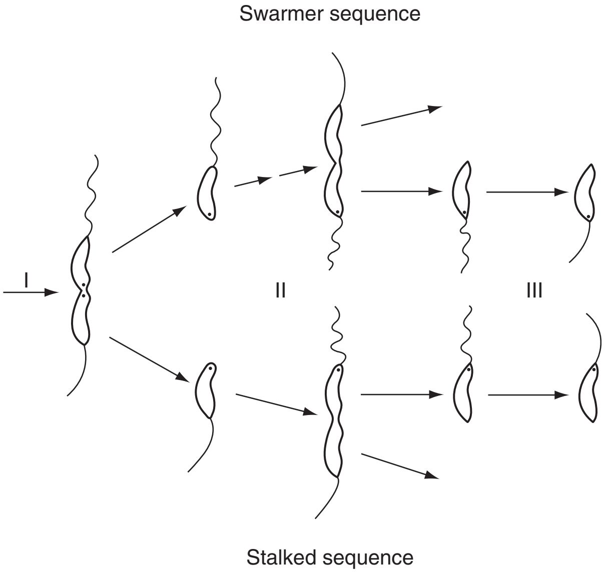

Figure 9 Sequence of pole development events in three successive generations. The sequences are displayed here in parallel to emphasize that the sequences are identical at the sibling poles formed by any one fission process. However, the swarmer sequence in generation II is typically about 1.33 times as long as the sequence occurring in its stalked sibling.

genomic and proteomic analyses, physiological studies, environmental manipulations, and studies of cellular composition and protein localizations. The research has yielded an elaborate description of the development of cell shape and of functional appendages in a unicellular bacterium whose unique morphology and cell cycle have been creatively exploited as a model system for the elucidation of bacterial cell biology. (For the reader interested in full-length versions, several reviews are listed in the Further Reading section.)

Fission and the Development of Cell Shape

Cell wall synthesis

The shape of a bacterial cell requires maintenance by a rigid element, which is a sacculus (see **Figure 5**) of peptidoglycan, sometimes called 'murein'. The best-known pathway of synthesis in a Gram-negative bacterium occurs at three locations in a Gram-negative bacterial cell:

1. In the cytosol: small-molecule precursors are synthesized by intermediary metabolism, then combined into precursors of the repeating unit of the cell wall polymer.
2. At and within the cell membrane: the saccharide pentapeptide is delivered to the inner, cytoplasmic surface of the cell membrane, transferred to a lipid carrier, and moved into the membrane, where a second saccharide unit is added to the lipid-carried precursor. This disaccharide-pentapeptide-lipid carrier is then conveyed through the membrane to its outer periplasmic surface.
3. On the outer surface of the cell membrane and in the periplasm: the disaccharide pentapeptide is transferred from the lipid carrier to an acceptor site (either an end recently synthesized or a position opened by a lytic enzyme) on the preexisting peptidoglycan of the sacculus in the periplasm. This process elongates the glycan backbone of the peptidoglycan as the lipid carrier is recycled within the membrane. Transpeptidases then strengthen the sacculus by crosslinking between the outer D-ala-D-ala pair of one pentapeptide and the free amino group on the diamino amino acid (most commonly diamino pimelic acid) of another glycan chain.

The details of synthesis vary among bacteria; nevertheless, the overall process is general for *Eubacteria*, and similar systems occur in some *Archaea.*

Peptidoglycan synthesis complexes can affect the shape of an elongated cell when they are active at three regions:

1. Along the length of the cell to support elongation along the cell's long axis. So far as known at present, the complexes are arranged as a helix in rods, vibrios, spirilla, and fusiform cells.
2. At midcell to provide new peptidoglycan for constriction or septation. So far as known at present, the complexes are arranged as a ring in this location.
3. At a cell pole to support polar outgrowth such as occurs in budding and/or prosthecate bacteria. A specific geometric pattern of installation has not yet been discerned.

Research on bacterial cell shape development is, in large measure, research on peptidoglycan synthesis, and is aided by the availability of natural and artificial products that specifically inhibit one or another synthetic step. Example of inhibitors used in studies of *C. crescentus* morphogenesis include cycloserine, which inhibits the formation of D-ala-D-ala from L-ala in the cytoplasm; vancomycin, which inhibits transglycosylation at the outer surface of the cell membrane; and β-lactam compounds such as amdinocillin that inhibit crosslinking in the periplasm. Van-FL, a derivative of the cell wall synthesis inhibitor vancomycin, binds to and detects the D-ala-D-ala moiety of uncross-linked peptidoglycan. It can be used to tag nascent peptidoglycan in bacteria with highly crosslinked peptidoglycan, particularly Gram-positive species. However, the peptidoglycan of *C. crescentus* is not highly crosslinked, and Van-FL tagging probably does not adequately distinguish sites of particularly active peptidoglycan synthesis from less active or even inactive sites.

Cytoskeleton

Three cytoskeletal proteins have been discovered in *Eubacteria* that appear to guide the placement of cell wall synthetic complexes. Each of these proteins is homologous to cytoskeletal proteins of eukaryotic cells:

1. FtsZ, a GTPase, is a tubulin homolog that participates somehow in cytokinesis (pole formation); in *C. crescentus*, it also participates in stalk initiation (pole outgrowth).
2. MreB is an actin homolog whose principal role appears to be to guide cell elongation.
3. Crescentin may be homologous to intermediate filaments of animal cells; it accumulates within the cytoplasm on the concave side of vibrioid cells and appears to generate cell curvature and possibly also twisting. Discovered in *C. crescentus*, its role in bacteria other than caulobacters has not yet been established, but similar proteins are anticipated.

The properties and functions of these cytoskeletal proteins have been investigated in rods such as *Escherichia coli* and *Bacillus subtilis*, and in staphylococci, and the research with *C. crescentus* has been particularly fruitful in elucidating the dynamic nature of their localization during the course of a cell cycle. In such research, caulobacter cells offer two marked advantages over other bacteria: the relative age of each cell's two poles can be inferred in microscopical preparations, and one synchronous developmental sequence can be followed by the procedurally simple means of differential centrifugation that segregates the nonstalked cells (swarmers) from their distinctly more buoyant stalked siblings.

Placement of cytoskeletal proteins and cell wall synthesis complexes

During the sequence of developmental events at a caulobacter pole that are observable by light and electron microscopy of whole cells, a submicroscopic sequence also occurs. This sequence has been revealed by microscopical methods that detect the presence of a specific protein within the cell with sufficient resolution to determine whether it is dispersed throughout the cell, restricted to the cell surface, or accumulated at a particular locale along the long axis. In *C. crescentus*, microscopical studies have revealed a hierarchy of protein placement dependence in which the accumulation of one protein at a site facilitates or is necessary to the subsequent accumulation of another protein.

These studies have employed a molecular biological toolkit of chemicals, mutants, and highly specific genetic manipulations, the details of which are beyond the scope of this article. They have enabled investigators to follow events in deletion mutants that cannot produce specific proteins, in strains that overproduce specific proteins, and others that can be depleted of specific proteins due to the cessation of synthesis or when a specific protein accumulation is dispersed by being depolymerized. Sequence, duration, and location of specific events are followed by the fluorescence of fluorochrome-carrying proteins or by protein-specific fluorescent antibodies. The studies have depended heavily on observations of the events in the swarmer sequence (see **Figure 9**), where synchrony is far tighter than in populations of stalked cells.

In *C. crescentus*, as in most bacteria so far examined, FtsZ is present at the site of constriction during cytokinesis. Associated with this ring are peptidoglycan-synthesizing complexes that contain MurG, the transglycosylase that adds the second sugar to the monosaccharide-pentapeptide-lipid carrier. The completed precursor is then flipped from the cytoplasmic to the periplasmic surface of the cell membrane, an activity currently attributed to RodA, an integral cell membrane protein. As constriction is completed, FtsZ is visible within each incipient pole. When the siblings separate, the polar FtsZ disappears due to proteolytic destruction that seems faster in the stalked than in the swarmer sibling.

As the swarmer sequence progresses, FtsZ synthesis resumes when the flagellum is shed and DNA replication begins (see section 'The core regulatory cascade of transcription regulation'). The new FtsZ appears first as a helix that extends the entire length of the cell within the cytoplasm. It is accompanied by MreB, also in a helix, and by a third helix, of MreC, in the periplasm. A fourth protein (this is not a full list) known to become part of this system is PBP2, a periplasmic transpeptidase. As the complexes mature, peptidoglycan synthesis can occur along the helices, and the cell elongates.

Early during DNA replication, the three membrane-associated proteins FtsZ, MurG, and MreB (at least) are directed to begin accumulation as a ring at midcell. As this region matures, the synthesis of peptidoglycan causes elongation from midcell toward the poles. At about the same time, a third site of synthesis is established at the older pole and initiates elongation of that pole as a stalk. The proteins apparently responsible for stalk elongation, which occurs only at the cell-stalk junction, include MreB and RodA. After the midpoint of DNA replication, constriction becomes apparent as the midcell site of peptidoglycan synthesis develops into two new poles. Meanwhile, because the swarmer's sibling lacked a motility period during which DNA synthesis and peptidoglycan synthesis at the pole were suspended, these events have already occurred in the stalked-cell sibling. In both siblings, cell wall synthesis at the cell-stalk junction appears to pause during cell separation, then resume, probably as soon as DNA synthesis resumes in the next cycle.

Two mechanisms could explain how a protein recognizes and positions itself at a particular location. Its structure could confer on it an affinity for another macromolecule, the target; such molecular recognition is one of the most important properties of proteins. Alternatively, it could encounter a barrier at some sites that prevents its close approach or binding; by default, it would then bind only at barrier-free sites. Both mechanisms are reasonable and precedented; both beg the question of what places the target or the barrier. So far, in *C. crescentus* studies of these peptidoglycan-shaping proteins, only FtsZ seems capable of finding its own way within the cell relatively independent of the other pole- and surface-localizing proteins involved in shaping the cell. Nevertheless, others in this set of proteins (particularly MreB) are required to lead the cell wall synthetic complexes to the FtsZ. In some bacteria, FtsZ rings can form only in areas within the protoplast that do not contain proteins that inhibit polymerization of FtsZ by stimulating its GTPase activity. These proteins also tend to be DNA-binding proteins that associate with specific genome regions so that their location is determined by the location, position, and state of replication of the DNA. Such proteins so far identified in *C. crescentus* include MipZ and ParB, which may provide a direct molecular connection between cell shape development during the cell cycle (most importantly, in this instance, constriction) and the DNA replication cycle.

What happens to a cell in which these events are deranged by chemical abuse, genetic modification, or malnutrition? Cell shape becomes bizarre – lemons rather than vibrios; abnormally wide stalks; stubby or branched or multiple or misplaced (ectopic) stalks; filamentous cells that divide infrequently; and so on. Specific aberrations help investigators interpret the function of a missing participant in shape determination or protein placement, as does recovery toward normal shape following relief of an inhibitory condition. Without or before relief, many cells disintegrate and die.

Extrusion Events at the Younger Cell Pole

‘Extrusion’ is used here to refer to the assembly of cell components, other than the typical outer membrane of a Gram-negative bacterium, that are located superficially, external to the cell membrane, and installed only at cell poles (except, of course, in *Asticcacaulis*; see section ‘Other caulobacters’). Freshwater caulobacters such as *C. crescentus* also produce and assemble a surface array, or S-layer, on the exterior of the outer membrane. However, that layer is made constantly through the cell cycle, covers the entire cell body and stalk surface evenly, and consists of a single major exported protein. Although it is presumably of great value to the cell and has been exploited in technological applications of *C. crescentus*, it is not described here because its production, assembly, and possibly its function are neither unique to caulobacters nor an aspect of the asymmetry of development in this bacterium.

Motility: Flagellum and chemotaxis proteins

Assembly and activation of a flagellum at the younger pole of a dividing cell can be observed as the appendage, its activity, or its component proteins. Preparation for the development of a new flagellum begins in the swarmer cycle as the swarmer’s original flagellum is shed, motility ceases, and stalk outgrowth and DNA replication begin. Within the cell, a transcription cascade of three sets of genes begins. The products of some of these genes regulate expression of other genes in the cascade, while others’ products are functional components of the flagellum apparatus. The first set (‘class II genes’) produce the motor switch and its ring; the second set (‘class III’) produce the basal body and hook; and the third set (‘class IV genes’) produce the flagellin subunits that are assembled into the rigid external helical filament whose rotation will propel the cell through its liquid environment. In the cascade, transcription of each successive class is required for the expression of the next class, and each set of proteins is assembled onto products of the previous set.

During the swarmer cycle, this regulatory system results in temporal (because of transcription dependence) and spatial (because of assembly dependence) ordering of the development of the next flagellum. Meanwhile, protein components needed for chemotactic sensors and response regulators are produced and accumulated in the incipient swarmer progeny as the cell begins to divide. The flagellum begins to rotate as constriction progresses at midcell, and the newborn swarmer is freely motile immediately upon separation from its stalked sibling. Within the constriction site, two proteins, TipN and TipF, accumulate that seem to mark each of the new poles as sites of flagellum assembly in the next cell cycle.

The final step in the life of a flagellum occurs in the next swarmer cycle at about the time that DNA replication begins: release of the flagellum from the older cell pole. This process is accompanied by proteolytic destruction of the motor switch protein FliF by a specific protease, ClpXP. While the mechanism of release is not yet certain, the appearance of the shed flagella that accumulate like litter in the liquid phase of laboratory cultures is consistent with a cleavage-effected separation. The filament, the hook, and the rod are still an intact unit, but the other membrane-associated components are absent.

Thus, the overall organization of the development of flagellar structure and function follow mechanistic principles similar to those of sacculus development, although regulation of transcription may play a somewhat larger role here. An immediate role for cytoskeletal proteins has not been demonstrated for flagellar morphogenesis, but it is clear that the positioning of the flagellar apparatus derives from the ultimate guidance of the constriction site placement by the cytoskeleton.

Adhesiveness: Holdfast and pili

Many kinds of bacteria – aquatic, terrestrial, plant and animal associates – adhere to bulky (bulkier than bacterial cells) substrata by means of some type of adhesive substance, most commonly composed of or containing a significant amount of polysaccharide. For mechanical and/or electrostatic reasons, initiation of adhesion is often facilitated by motility of the bacterium. In many bacteria, it is also aided by sticky protein filaments (pili or fimbriae) that extend from the cell surface and enlarge the effective diameter of the cell, increasing its frequency of contact with other particles.

The younger pole of a caulobacter cell is provided with all three devices for establishing persistent, stable adhesion to substrata. At about the same time that the new flagellum becomes visible and active on a dividing cell, extrusion of adhesive material (the 'holdfast') occurs at the same pole. The glue is composed of poly-*N*-acetylglucosamine plus other components not yet identified. It is the strongest adhesive known among biotic glues, stronger than the adhesives of marine mollusks. It works under water and allows caulobacters to adhere to a wide diversity of organic and inorganic inanimate materials and to other microorganisms, most commonly algae and cyanobacteria in natural samples, without damaging the substrate cells. Once sessile, a caulobacter cell remains fixed in place; there seems to be no mechanism for the cell to reverse adhesion, and the holdfast seems to retain its adhesiveness indefinitely.

In several species of caulobacters, pili are also extruded by assembly at the same pole as the flagellum and holdfast, but unlike those structures, pili are not universal among isolates. In *C. crescentus*, the pili accompany the other polar structures in position and in time during the cell cycle.

> Caulobacters are fun to watch. Prior to sibling separation, if the stalked pole is not attached to the slide or cover slip, the single flagellum drives the dividing cell stalk foremost. The flagellated pole can attach without causing cessation of flagellar rotation, swinging its stalked sib around with the stalk waving in the liquid in a direction that reveals that, most of the time, flagellar rotation is counterclockwise. When the siblings separate with neither of them attached to a substratum, the swarmer pauses briefly and then resumes swimming at a speed noticeably greater than it achieved while it carried the additional mass of its stalked sibling.

One seemingly awkward aspect of adhesion initiated by the swarmer is direction: the flagellum drives the cell predominantly with the adhesive, flagellated pole as posterior and the newborn, nonadhesive pole in the lead. While this should prevent adhesion to most of the particles the cell approaches, motility should assist adhesion only when the cell backs up.

In most caulobacters, the three structures important to adhesion arise at the site on the younger pole of the dividing cell that will grow outward as the stalk. In the closely related genus *Asticcacaulis*, stalk outgrowth occurs at a separate site, in one species not even at the pole (see section 'Other caulobacters' and **Figures 4** and **7**). As the flagellum is shed and stalk outgrowth begins, the pili are retracted by depolymerization within the periplasm, but the holdfast remains on the tip of the growing stalk. In contrast to stalk outgrowth, which probably pauses during cytokinesis and certainly continues (or resumes) in each cell cycle, synthesis and extrusion of the holdfast, pili, and flagellum occur only once in the life of a pole. If the cell does not adhere to a substratum while motile, it is much less likely to do so after it sheds its flagellum and begins to develop a stalk, even though the holdfast persists. It can migrate to a new locale only through its swarmer progeny. Given such a long-term commitment to a substratum, is there any mechanism that promotes or guides adhesion of the cell to a favorable environmental site?

The answer presumably lies in the ability of the cell to move to a favorable location chemotactically. Like other motile bacteria, caulobacters are provided with a chemical gradient sensory system that influences the activity of the flagellar motor. Unlike peritrichously flagellated bacteria, these monoflagellate swarmers do not tumble, but they do occasionally stall. Brownian motion is sufficient to reorient the cell during a stall, and this passive tumbling results in a redirection of the cell's swimming when rotation resumes. The components of the chemotaxis system, synthesized before midcell constriction occurs, are placed at the cell poles. However, even before constriction is completed, they are swiftly destroyed by proteolysis at the stalked pole and so are inherited almost exclusively by the swarmer sibling. Proteolysis eventually eliminates the system in the swarmer sibling as it begins the transition from swarmer to stalked cell, in concert with the shedding of the flagellum.

In the limited studies of specific stimuli of chemotaxis in caulobacters, the strongest influences on this response have proved to be potential nitrogen sources. This is consistent with the striking effect of nitrogen limitation as an inhibitor of the events that begin the maturation of a swarmer into a stalked cell. It implies that a major purpose of motility in these oligotrophic bacteria is to reposition the young swarmer not only away from its sibling and into an aerated region, but toward a substratum fixed in a nitrogen-adequate location.

Bacteriophage receptors

There is a final set of functions that are transiently present at the younger pole of a dividing cell: reception of bacteriophage. With few exceptions, the attachment of bacteriophage produced by caulobacters is pole-specific, and the pole is the same for all such viruses - the flagellated, sticky pole. All the RNA phages of caulobacters attach to the pili; at least one type of DNA phage attaches to the flagellum; and the majority of DNA phages attach directly to the pole, either to unidentified components of the outer membrane or to protein(s) situated at the site through which pili are extruded. None is known to be holdfast-specific. The unusual consequence of this pole specificity is that bacteriophage infection is a 'childhood' disease of caulobacters, in the sense that susceptibility to infection depends on traits of the juvenile stage, the swarmer. As a consequence, virus infection spreads through a caulobacter population only when the environmental conditions are suitable for completion of at least one reproductive cycle – and for the production of virions.

Influence of Macronutrient Availability on Stalk Initiation and Elongation

Almost all of the nutritional experiments have been conducted with the species *C. crescentus*, which is amenable to the studies of nutritional influences because it can grow in chemically defined media. Nevertheless, one principle regarding development in caulobacters is observable with every isolate tested: development responds significantly to changes in nutrient concentration, even in complex media. Phosphate and nitrogen-source effects were recognized early; effects of carbon source, calcium, and magnesium are also known, but have received relatively little attention to date.

Nitrogen source and stalk initiation

In general, caulobacter isolates can be cultivated in complex media containing peptones, which provide nitrogen as amino acids and peptides. *C. crescentus*, *C. leidyi*, and some *Asticcacaulis* isolates can be cultivated in media that provide inorganic nitrogen as ammonium or nitrate salts. However, because *C. crescentus* lacks the low-affinity ammonium uptake system of glutamate dehydrogenase and depends on the high-affinity GS/GOGAT system for ammonium assimilation, it starves for nitrogen when ammonium is in excess. Such starvation is relieved by supplementation of the medium with glutamate or by substitution of nitrate for ammonium. Nitrogen limitation has one long-known, demonstrable effect on the cell cycle: nitrogen unavailability causes cell cycle arrest that postpones release of the flagellum, onset of DNA replication, and initiation of stalk outgrowth. Nevertheless, little attention has been paid to nitrogen availability in many of the developmental studies; the molecular details of development so far described are based on studies of development under nitrogen-adequate conditions.

Phosphate and stalk elongation

In considering stalk length in caulobacters, the age of the stalk, which grows during each cell cycle, needs to be accommodated. First-generation stalks have had only one growth period, third-generation stalks have had three growth periods, and so on. To compare two differently manipulated populations with each other with respect to stalk length, results will be informative only if stalk length is measured only for the stalks in the same generation. The first generation is by far the most variable, probably due to composite influences on two stages of stalk development – stalk initiation and stalk elongation. After the first stalk band has been installed, stalk outgrowth occurs by stalk elongation only, and that process can be evaluated separately from stalk initiation.

Phosphate concentration in finite cultures and phosphate flux in perpetual cultures (chemostats) exert striking effects on stalk development. At high phosphate concentrations or fluxes, stalk length by the end of the second cell cycle does not significantly exceed average length of siblings at the time of completion of cell constriction. During phosphate exhaustion in a phosphate-limited finite culture and most of the time in a phosphate-limited chemostat culture, second-generation stalks (with one or two bands) are several times longer than newborn cells. It is readily inferred that abundant phosphate retards stalk elongation. It probably also inhibits stalk initiation, at least in mutants that appear 'stalkless' until grown in low-phosphate conditions. Stalk initiation is, however, difficult to recognize until it is followed by stalk elongation, and so cannot be studied independently of elongation.

Like many other bacteria, *C. crescentus* possesses a Pho regulon of genes whose expression is governed by the availability of phosphate. Most of these genes occur in an operon containing at least three genes for high-affinity phosphate transport proteins and at least three for sensing and responding to the availability of phosphate; another transport gene, *pstS*, is at a separate locus. If these genes work together in a manner similar to such genes in *E. coli* (which seems probable), the relationship between phosphate abundance and rate of stalk elongation would be as follows.

1. The proteins PstC, PstA, PstB, and PstS form a cell membrane complex capable of high-affinity phosphate transport. The complex is associated with the peripheral cytoplasmic protein PhoU, and through PhoU with the integral membrane protein PhoR. PhoU is a sensor protein and PhoR is a response modulator. The response regulator, PhoB, is cytoplasmic, not membrane-associated.
2. When phosphate is abundant, PhoR remains with the complex, but when phosphate is scarce, it is transported via the Pst complex. The passage of phosphate

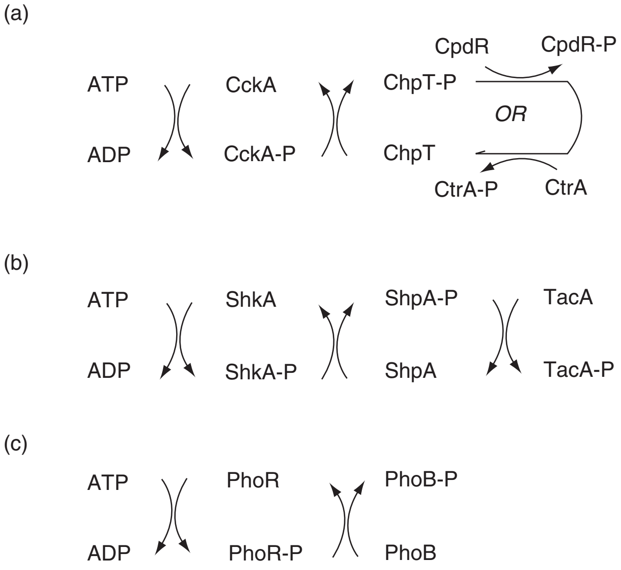

Figure 10 Example phosphorelays (P-relays) known to influence pole development and progress through the cell cycle in *Caulobacter crescentus*. (a) CckA-initiated, to the gene activator CtrA-P. (b) ShkA-initiated, to the gene activator TacA, which can also be phosphorylated via CtrA-P. (c) PhoR-initiated under conditions of phosphate limitation, to the gene activator PhoB-P.

through the complex releases PhoR from the complex, but not from the cell membrane. PhoR then autophosphorylates (see **Figure 10(c)**) and transfers its phosphate group to PhoB.

3. PhoB-P activates genes of the Pho regulon, increasing the abundance of the Pst proteins and providing other features for dealing with phosphate limitation, such as synthesis of phosphatases that enable the cell to consume phosphorus from organic phosphate compounds. In *C. crescentus*, these events are accompanied by a marked acceleration of stalk elongation.

Studies with cell wall synthesis inhibitors indicate that some of the immediate effectors of stalk outgrowth (initiation and elongation) catalyze peptidoglycan synthesis and/or modification, but no such enzyme has yet been identified as unique to stalk sacculus development. Studies of mutants that seem able to divide without stalk outgrowth when cultivated in phosphate-rich media have consistently been found able to initiate at least some outgrowth when subjected to phosphate limitation. Decades of searching for *C. crescentus* mutants that are viable yet lack at least one piece of genetic information essential to stalk development has not yet yielded such a mutant. Every caulobacter laboratory has recognized the implication that because genetic stalklessness appears to be lethal, it is reasonable to regard stalk development as a process of cell surface morphogenesis that is common to pole outgrowth and pole formation. The latter process cannot be lost without eliminating fission. Nevertheless, it is not unreasonable to expect that some step late in the hierarchy of regulation of stalk development could be eliminated without preventing fission, and a viable unconditionally stalkless mutant may yet be found.

Proteomic characterization of the *C. crescentus* stalk has identified several proteins that are known to participate in nutrient acquisition – for example, TonB-dependent receptors, certain outer membrane pore/channel proteins, and phosphatases and other hydrolytic enzymes that assist in increasing the transportability of charged substrates. Altogether, the stalk proteins so far identified appear to be a subset of the cell body periplasmic and outer membrane proteins; none appears to be unique to the stalk. Whether any of the stalk proteins are installed in the stalk only during phosphate limitation has not yet been systematically explored.

Control of Development: Protein Synthesis, Modification, Localization, and Destruction

The core regulatory cascade of transcription regulation

For the first three decades or so of developmental research in *C. crescentus*, attention focused intensely on determining the cause of the postponement of developmental events in the swarmer sibling – events that occur in the stalked sibling in the same order and at the same rate, but suspended during motility in the swarmer sibling. By the early 1990s, a lengthy description of regulation through transcription control, protein phosphorylations that alter protein activities, and installation of specific proteins at specific cellular locations had been elucidated. Advances since then in genome manipulation, sequencing, and interpretation, as well as in subcellular microscopy of living cells and in physicochemical analysis of protein structure, have greatly accelerated the elucidation of further details.

The core of the cell cycle's direction as described by about 1990 has proved adequate as new information has been fit into that core. Following is a brief description of the major regulatory features that govern development in both siblings: swarmer and stalked cell.

Four regulatory proteins constitute the core of the regulatory mechanism: CcrM, DnaA, GcrA, and CtrA. The last three of them are transcription regulators that interact with each other's genes and *ccrM*; as a group, they also control at least 200 other genes whose products mediate the cell changes and assist in regulation. Most of the principles of cell cycle regulation in *C. crescentus* can be laid out in terms of these four proteins – their regulated expression, their placement relative to the cell poles, and their cyclical destruction by proteolysis.

Description of a cycle can begin at any point; the point used here is the epigenetic step: methylation of specific nucleotides close to a gene's promoter that affects the gene's transcription. Each of the other regulatory proteins activates or inhibits transcription.

1. CcrM is the DNA methylase: its methylation of DNA activates *dnaA*, but inhibits expression of *ctrA* and of its own gene, *ccrM*.
2. DnaA activates *gcrA*: through a multitude of other genes in its regulon, DnaA is essential for initiation of DNA replication.
3. GcrA (probably not alone) activates *ctrA* transcription from the first of *ctrA*'s two promoters, P1 and P2.
4. CtrA is phosphorylated by the CckA-initiated P-relay (see **Figure 10**(**a**)). CtrA-P has multiple regulatory effects as it accumulates:
    1. activation of *ctrA* through its second promoter, P2, providing positive feedback regulation of its own transcription;
    2. inhibition of *ctrA* transcription through P1, providing negative feedback of control of its transcription by GcrA;
    3. inhibition of transcription of *gcrA*; a, b, and c together shift responsibility for regulation of *ctrA* from GcrA to CtrA-P;
    4. prevention of initiation of DNA synthesis by binding near the origin of replication (*ori*), blocking the binding of the replication proteins. Initiation of DNA replication happens only once in each cell cycle in *C. crescentus*, necessarily occurring before CtrA-P accumulates;
    5. activation of *ccrM*, reinitiating this regulatory cascade by activating DNA replication by inhibiting transcription of *ctrA*.

The operation of this cascade results in the sequential transcription of the four genes in order: *dnaA*, *gcrA*, *ctrA*, *ccrM*. Each of the gene products DnaA, GcrA, and CcrM is effective as an unmodified protein and begins to function as it accumulates. CtrA, in contrast, must be converted into CtrA-P by phosphorylation, which is the role of a phosphorelay (P-relay) initiated by an autophosphorylating kinase, CckA.

Roles of phosphorelays

Protein phosphorylation is a mechanism for controlling the activity of catalytic proteins, which may be activated or inactivated by the addition of one or more phosphate groups to target sites, typically serine, threonine, or histidine residues. Cells also possess phosphatases that remove the phosphate groups and reverse the direction of control. P-relays provide control of metabolism by making the phosphate groups available to the families of proteins whose activation-inactivation depends on the presence and activity of the relay components; the relays thereby serve as a buffer to balance activation among pathways and their branches and so to direct metabolic traffic. Operation of a P-relay requires, at a minimum, a kinase that transfers the phosphate group ('∼P') from a nucleotide triphosphate to a protein (including itself, if capable of autophosphorylation), and a phosphotransferase to move the ∼phosphorus to at least one other protein. In a P-relay involved in regulation of transcription rather than of enzyme activities, one of the proteins along the relay is converted from an inactive form into a gene-activating protein that will target promoters that contain a unique, shared sequence. Activation of a set of such genes (a regulon) provides the cell with new, usually related properties simultaneously.

The genome of *C. crescentus* contains 106 genes – an exceptionally high number – for two-component signal-transduction proteins that function via P-relays. The activity of one-third of these genes rises and falls as the cell progresses through the cell cycle, implying that they participate in the regulation of morphogenesis in *C. crescentus*. Such roles are known for two of the three examples of *C. crescentus* P-relays illustrated in **Figure 10**. The CckA-initiated P-relay (**Figure 10**(**a**)) passes phosphate groups from ATP to ChpT, a branch point protein in the relay from which the ∼phosphorus can be passed to more than one other protein. One of those proteins is CtrA; another is CpdR. The CckA-initiated relay is, therefore, integral to the core of cell cycle regulation in *C. crescentus*.

The second relay illustrated (**Figure 10**(**b**)) transfers ∼phosphorus from ATP through ShkA (the kinase) to ShpA (the phosphotransferase) and then to TacA (the response regulator). TacA-P is an enhancer-binding protein that can also be phosphorylated through CtrA-P. One of the genes activated by TacA-P, *staR*, is essential for stalk elongation in nutrient-rich medium; the requirement for *staR* may account in part for the known need for intact *tacA* for that process. The third, shortest relay (**Figure 10**(**c**)) illustrates the role of a P-relay in the response of Gram-negative bacteria, presumably including caulobacters, to environmental limitation for phosphate as a nutrient, which was discussed above (see section ‘Phosphate and stalk elongation’).

Chromosome and protein localization

In the dividing cell, one copy of the origin of replication (*ori*) of the *C. crescentus* chromosome is situated at each pole. Completion of constriction separates the siblings, each with a copy of *ori* at its now-older pole. In the next cycle, as soon as replication begins (sooner, in the stalked sibling; later, in the swarmer cycle), one copy of *ori* migrates to the younger pole. Thus, for most of the cell cycle, begun either by a swarmer or by a stalked cell, the two copies of *ori* are at opposite poles.

Also prior to the completion of cell fission, the protein CpdR has somehow guided the installation of the protease ClpXP at the stalked pole. Several other proteins become localized to the poles late in the cell cycle. Motility-associated proteins of the entire flagellar structure and chemotaxis system (e.g., McpA) are found only in the swarmer sibling, some installed in the cell envelope, others dispersed in the cytoplasm. Proteins associated with constriction (including FtsZ, MurG, and RodA; see ‘Placement of cytoskeletal proteins and cell wall synthesis complexes’) have gathered at about midcell. By the time the cell divides into two morphologically distinguishable siblings, they are also distinguishable by the presence of and arrangement of internal proteins. Probably most significantly, the core regulatory protein CtrA remains at *ori* within the stalked pole, but disperses into the cytoplasm in the swarmer sibling.

Starting over: Proteolysis

Following the separation of the two copies of *ori*, CtrA-P accumulates, binds to *ori*, and blocks initiation of another round of replication during the remainder of the cell cycle, in both siblings. However, during constriction, while newly synthesized CtrA remains dispersed in the swarmer compartment, CtrA becomes localized at the stalked pole in the other compartment. Also localized at the stalked, but not the flagellated, pole is a complex of proteins that includes the protease ClpXP. Within that complex, ClpXP destroys the local CtrA and frees *ori* for the initiation of DNA replication.

An efficient and dependable way to halt the core regulatory cascade and reset the entire developmental sequence would be to destroy at least one of the core proteins. Which one to destroy?

- CcrM? CcrM destruction would not be efficient because CtrA-P promotes CcrM synthesis and would compete with proteolytic removal of CcrM.
- GcrA? By the time CtrA-P appears, GcrA has already lost control of *ctrA* transcription, so it is no longer part of the cascade.
- DnaA? DnaA destruction would prevent DNA initiation, which is already blocked by CtrA-P.
- CtrA/CtrA-P? CtrA destruction would allow residual CcrM to promote synthesis of DnaA, enabling DNA synthesis; it would clear away the blockage at *ori* so that DNA replication could be initiated; and *ctrA* transcription would be brought back under the control of the cascade. Finally, CtrA phosphorylation by the CckA-initiated P-relay (**Figure 10**(**a**)) would lack its CtrA substrate.

C. crescentus destroys the CtrA. The responsible protease is positioned at the stalked pole of the dividing cell and does not diffuse into the incipient swarmer. Adding to the asymmetry of the next events is a role of the CckA-initiated P-relay, which is interrupted in the stalked sibling. As phosphorylation of CtrA fails, the protein becomes more susceptible to proteolysis and is destroyed, and the cascade begins again with the production of CcrM.

CtrA-P persists for a while in the newborn swarmer, but proteolysis of CtrA will occur in the swarmer sibling later – after the period of motility – and involves proteins and events that are not yet as certain as those identified in the stalked sibling. There are roles for the CckA-initiated P-relay, a phosphatase (PleC) localized in the swarmer cytoplasm, DivK/DivK-P, and once again for ClpXP. In addition to the effects of CtrA-P noted above, CtrA-P activates *divK*, whose product inhibits the P-relay from CckA to CpdR. Inhibition of CtrA proteolysis, an effect of CpdR-P, probably declines, allowing CtrA proteolysis in the swarmer, finally resetting the core regulatory cascade. This seems to coincide with the release of the flagellum, retraction of the pili (if present), initiation of DNA synthesis, and the onset of stalk outgrowth.

Besides CtrA, ClpXP also attacks FliF, the flagellar motor switch, and McpA, a chemotaxis protein, both located at the flagellated pole. Proteolysis of FliF is a candidate as a crucial step in the release of the flagellum, which occurs close in time to the onset of DNA replication. The McpA will be replaced by renewed synthesis as the transcription cascade of flagellar genes begins again later in the cell cycle.

In summary, although the story of development in this dimorphic bacterium is a 3-chapter tale of poles, the cast of participants includes scores of proteins. The plot involves regulation of transcription; covalent modification of proteins that alters their activities; and localization of cytoskeletal proteins, cell wall synthesis complexes, DNA-binding proteins, and structural proteins to target sites or away from barriers. Unraveling the plot has revealed that these bacteria achieve functional compartmentalization of the protoplast by localization of proteins rather than by the familiar elaboration of internal membrane barriers seen in eukaryotic cells. Ultimately, degradation of certain participants resets the sequence and renews the cycle of events. The greater the detail in which this story is related, the stronger the implication that the unique dimorphy of caulobacters expresses a complex and sophisticated genetic heritage, and that dimorphy is of great significance to the success of caulobacters as they eke out their existence by competing for nutrients that are perpetually scarce.

In today's biology, we accept the notion that cells do not create genes; they copy them. The remarkably detailed molecular studies of *C. crescentus* development should help us recognize the parallel throughout the cell: some molecules in addition to DNA must be present for still other molecules to be synthesized and ordered. Like peptidoglycan and DNA, which can be polymerized only in a cell that has some of each, the cascades, feedback loops, protein phosphorylation pathways, and other controlling events, which occur during *C. crescentus* development, occur normally only in cells in which certain key events are already in progress and where certain key proteins are already in place. *C. crescentus* research, with an abundance of molecular detail, is contributing to cell biology an elegant lesson in the meaning of the biotic continuum.

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Cell Cycles and Division, Bacterial

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Glossary

cell division genes Genes that encode for proteins that specifically function during the division process.

constriction, septation Mode of cell envelope invagination during division. During constriction all envelope layers move inward simultaneously and the daughter cells move gradually apart; septation involves the ingrowth of the cell envelope forming a T-like structure.

cytokinesis The process of cell division in prokaryotes. By contrast, in eukaryotic cells division also includes mitosis.

dcw *cluster* The cluster of genes involved in division (*d*) and cell wall (*cw*) synthesis. In many bacteria this cluster is evolutionary conserved.

divisome The macromolecular complex that carries out division at the cell center.

Fts proteins Cell division proteins encoded by *fts* genes. In temperature-sensitive (ts) cell division mutants, division is blocked, and because cells continue to grow filaments (f) are formed.

minicells Small DNA-less cells that arise through divisions at the poles of rod-shaped bacteria.

multifork replication More than one round of DNA replication going on in the same chromosome. It arises when the doubling time of the culture is shorter than the duration of the DNA replication period.

penicillin binding proteins Proteins involved in peptidoglycan assembly outside the cytoplasmic membrane. They bind specific antibiotics.

peptidoglycan Covalently closed structure that has the shape of a bacterium. It is composed of glycan chains, which have peptide side chains. Cross-linking through the peptide side chains provides for a strong network.

potential division sites Cellular sites beyond the nucleoid areas. They occur at cell pole or in the cell center, provided that the nucleoids have segregated.

Abbreviations

f	filaments
IM	inner membrane
OM	outer membrane
PG	peptidoglycan layer
PIPS	PBP3-independent peptidoglycan synthesis
ssDNA	single-stranded DNA
ts	temperature-sensitive

Defining Statement

The cell cycle of bacteria and archaea is treated with emphasis on the former. The difference with the eukaryotic and bacterial cell cycle is emphasized. The temporal relationship of the DNA replication cycle and the division cycle is elucidated. The division process is described in molecular terms.

Introduction

Proliferation of cells, whether pro- or eukaryotic, requires duplication of cytoplasm and genetic material (DNA) and the subsequent distribution of the new genomes over two daughter cells. For the individual cell this is achieved by fission of a cell that is on average 2 times as large as a newborn one. All molecular components should have been duplicated before fission.

Terminology and Concepts

Though the above description applies to all cells, some prokaryotes (bacteria) show features that are essentially different from their eukaryotic counterparts. It is important to appreciate these differences because, as will be shown below, they bear on concepts pertaining to the progression of the cell cycle. For instance, is a newborn cell completely new or is there actually a cell cycle?

According to the standard view, a eukaryotic cell cycle is punctuated by four sequential periods: the G1-period in which cells grow and prepare for DNA replication, the S-period in which DNA replication takes place, the G2-period where cells prepare for mitosis, and finally, the M-period during which chromosomes become compact, align in the cell center, and segregate producing new daughter cells. The fission of cells is termed cytokinesis. Note that in the eukaryotic field cell division encompasses mitosis and cytokinesis, whereas in prokaryotes cytokinesis is commonly designated as cell division alone. Of the many prokaryotic species, only a few bacteria have been or are being studied in detail. However, cell cycle studies on archaea are emerging. For bacterial cell division, a distinction should been made between Gram-positive (*Bacillus subtilis*, *Enterococcus hirae* (formerly called *Streptococcus faecalis* and *S. faecium*), *Streptococcus pneumoniae*) and Gram-negative species (*Caulobacter crescentus* and *Escherichia coli*).

Gram-positive organism divide by septation and Gram-negatives by constriction (**Figure 1**). In the Gram-positives, a circumferential inward-growing septum divides the new daughter cells, whereas in the latter, the cell constricts in the cell center (**Figure 2**). The cell envelope of *E. coli*, as in all Gram-negatives, is composed of three layers. From outside to inside these are the outer membrane, the peptidoglycan or murein layer, and the inner or cytoplasmic membrane. *B. subtilis*, as a typical Gram-positive, lacks an outer membrane (which affects the Gram stain) and has a thick cell wall composed of peptidoglycan and teichoic acid as the main components. Because of its rigid nature, the peptidoglycan-containing cell wall serves as an exoskeleton, which maintains the cell shape. Its strength prevents cellular disruption due to osmotic pressure and also plays an active role during the constriction process. Recent research has revealed an endoskeletal helix composed of the actin-like protein MreB directly underneath the inner membrane. Disruption of the helix leads to spherical cells, suggesting a functional interplay between MreB helix and peptidoglycan assembly in establishing and maintaining cell shape.

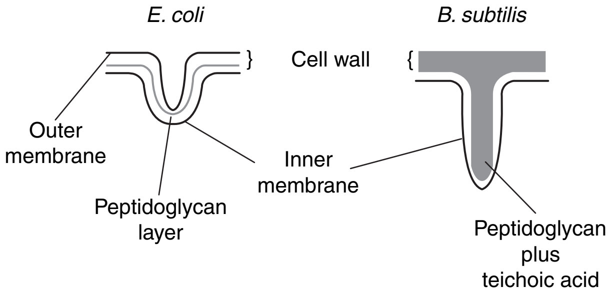

Figure 1 Division by constriction in *Escherichia coli* and by septation in *Bacillus subtilis*. During the constriction process the envelope layers invaginate together; during septation a T-structure is formed.

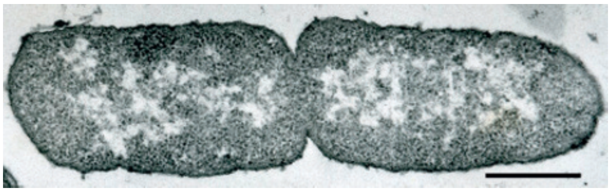

Figure 2 Electron microscopical image of a thin section of dividing *Escherichia coli*. Cytokinesis involves contriction of the envelope layers (cf. **Figure 1**). Scale = 0.5 μm. Courtesy of Dr. CL Woldringh.

To date, *E. coli* has been studied most intensively with respect to the cell cycle, though closely followed by *B. subtilis* and *C. crescentus*. For this reason, we will focus on *E. coli*.

However, some interesting features have emerged from the study of archaea, which differentiate them from bacteria and shed a broader light on prokaryotes in general.

The Cell Cycle of *E. coli*

The Cell Cycle Periods of *E. coli* Differ from those of Eukaryotes

How do the standard eukaryotic cell cycle periods compare with those of a bacterium like *E. coli*? In both cases, the lengths of the periods are affected by growth conditions. In eukaryotes the duration of G1 is directly related to the richness of the growth medium, the other periods being somewhat independent; however, in *E. coli*, the growth conditions specifically affect the position of the DNA replication period in the division cycle. In slowly growing cells, that is, cells growing in a relatively poor medium, an equivalent of a G1-period can be distinguished. This is followed by an S-like period denoted as C. However, a striking difference with eukaryotes can now be observed; in *E. coli,* DNA replication and DNA segregation go hand in hand. Therefore, in fact, S = M. Consequently, bacteria lack classical mitosis. In *E. coli* the C-period is followed by the D-period, being defined as the period between termination of DNA replication and cell division. During the D-period cells prepare for and carry out cytokinesis. Thus, the G2- and M-periods have no direct counterparts in *E. coli*. As will be shown below, archaea appear to resemble eukaryotic cells.

The Division Cycle and the DNA Replication Cycle Do not Coincide

The duration of C is affected by growth conditions. *E. coli* B/r cells that grow with doubling times (Tds) of 20–60 min at 37 °C have a C-period of 40 min and a D-period of 20 min. This length of D appears to be the minimal time span required to build the division apparatus or divisome (see below). Similarly, the shortest duration of C is about 40 min. Thus, under given growth conditions at least 60 min (C + D) have to elapse before fission is completed. Application of this rule for a range of doubling times is graphically presented in **Figure 3**.

Let us start with a Td of 60 min. Since C + D = Td, DNA replication must start at cell birth. At slower growth rates (Td > 60 min) a period without DNA synthesis after birth is seen, and it becomes longer as Td increases. In the example, the period is 20 min (**Figure 3**). When C + D < Td, for instance, when Td is 50 min the duration of the division cycle is not sufficient to complete DNA replication and division. The problem is solved by DNA replication beginning in the previous cycle. It can be seen that DNA replication has to start 10 min before cell birth (**Figure 3**). It will be appreciated that this leads to a newborn cell whose chromosome is one quarter replicated. Conceptually, this has an important consequence: a newborn cell does not start out with a new round of DNA replication.

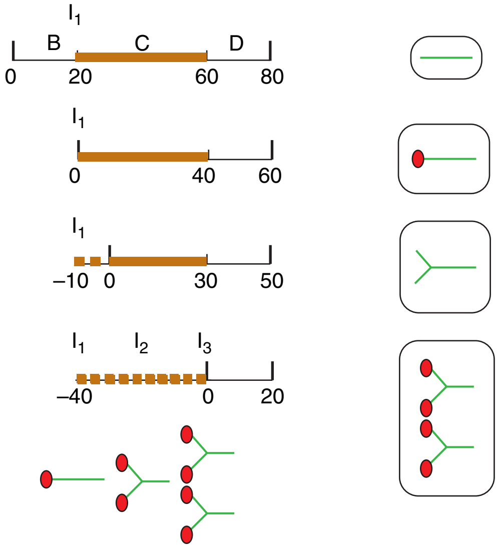

Figure 3 Temporal relationship between DNA replication period (C) and the division cycle in *Escherichia coli* B/r at doubling times (Tds) from 20 to 80 min at 37 °C. B, period between cell birth and initiation of DNA replication. D, period between termination of DNA replication and cell division. The C-period is shown as a brown bar. An interrupted brown bar denotes DNA replication before cell birth at the extant cycle. In this scheme, C = 40 min and D = 20 min. I_1, initiation of DNA replication belongs to the extant division cycle. I_2 and I_3, subsequent initiations of DNA replication. The frequency of initiation equals Td. The green line depicts an unreplicated chromosome. The red dot represents an activated origin of replication. At Td = 50 min, one quarter of the chromosome has been replicated at birth. At Td = 20 min, a newborn cell contains two chromosomes that have been replicated halfway. Initiation of DNA replication follows immediately after birth at the four origins. This happens before the ongoing round of replication has been completed. This results in multifork replication (cf. **Figure 4**). How one arrives at the chromosome configuration at cell birth is shown below. At Td = 20 min, 20 min are needed for the preparation of division (D-period), which is preceded by C = 40 min. Thus I_1 begins at –40 min as indicated. After 20 min at –20 min the chromosome is replicated halfway. At the same time (after one Td) a new round of DNA replication starts at I_2. One Td later chromosomes have segregated and are at the same time replicated halfway. New initiations start at I_3. Note that cells become bigger as Tds decrease. C and D values apply for *E. coli* B/r grown at 37 °C. For K12 strains they can be different.

Multifork Replication

What happens if C ≤ Td? For example, if Td = 20 min (when C + D = 60 min), DNA replication initiates at cell birth two cycles earlier (I_1 in **Figure 3**). A new initiation takes place every 20 min in line with Td. These subsequent initiations have been indicated at I_2 and I_3 in **Figure 3**. As a result, the newborn cell of the extant cell cycle has its chromosome not only fully replicated (two chromosomes in a newborn cell) but it also has been engaged in DNA replication for 20 min. Moreover, a new round of DNA replication starts at birth (I_3 in **Figure 3**). As a consequence, 5 min after birth, the bacterial chromosome is involved in two DNA replication cycles simultaneously. This phenomenon has been termed multifork replication (**Figure 4**).

In eukaryotes, multifork replication does not occur, presumably because chromosome compaction during mitosis precludes DNA replication. It is also for this reason that in eukaryotes the DNA replication cycle is contained within the ongoing division cycle. In bacteria, as outlined above, this is not obligatory. The fact that in bacteria the DNA replication cycle and its division cycle do not always coincide implies (as mentioned above) that a newborn bacterial cell is not completely new. Clearly, bacteria can also do without an identifiable G1-period. This has led to the notion that bacteria continuously prepare for the initiation of DNA replication, not only during DNA replication but also during D. It implies that the bacterial G1-period has no specific functional meaning; it simply arises because C + D < Td. It has been argued that the same reasoning would apply to the G1-period in eukaryotes. This common concept for pro- and eukaryotes has been called the continuum model by Cooper. The division cycle can be conceived of as a means for a cell to maintain and enlarge its molecular

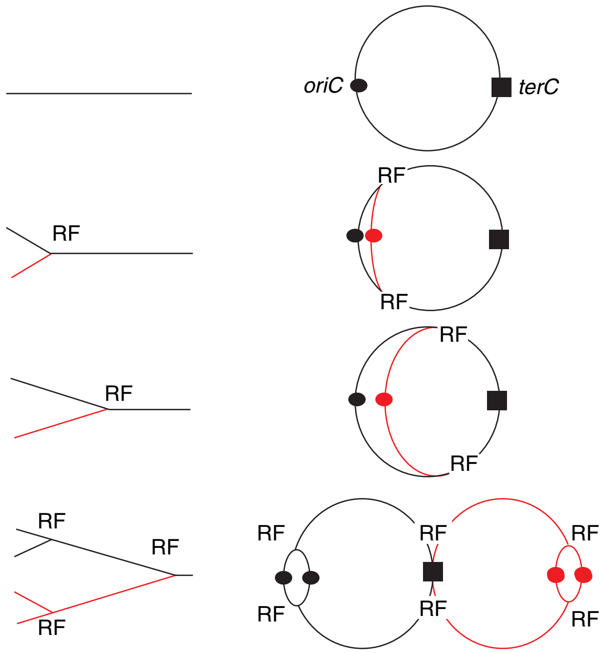

Figure 4 Linear (left, cf. **Figure 3**) and circular (right) representation of a replicating chromosome. oriC, origin of DNA replication; terC, terminus of DNA replication; RF, replication fork. The circle shows bidirectional replication. Below, multifork replication is shown.

fabric to allow replication of its genome. Thus, while mass continues to be made, the bacterial chromosome replicates, independently of division. This leads to the question, do cells really cycle?

As indicated above (**Figure 3**), cellular DNA content increases with the growth rate. Roughly, there is a relationship between cell size and the number of chromosomes that initiate DNA replication. Thus, dependent on growth conditions there can be a dramatic difference in cell mass and DNA content (**Figure 5**).

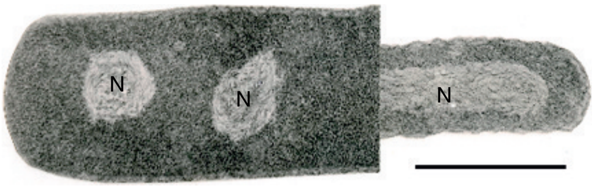

Figure 5 Electron microscopical images of thin sections of two *Escherichia coli* cell halves. The left and right cells have been grown at Tds of 21 and 150 min, respectively. The mean respective genome equivalents are 4.6 and 1.2. The left cell contains four replicating chromosomes at division, the right cell one. Scale = 1 μm. N, nucleoplasm.

DNA Replication Cycle

DNA Replication Cycle in *E. coli*

In the standard description of the eukaryotic cell cycle the G1-period encompasses protein synthesis including preparation for DNA replication during the S-period (see, however, the continuum model above). A separate description of the bacterial equivalent (B-period) does not seem warranted because it is often not distinguishable (dependent on growth conditions; see above).

It has been early recognized that initiation of DNA replication takes place at a defined cell mass (initiation mass). Of course, the challenge has been to translate this rather vague notion into molecular terms. In *E. coli*, the protein DnaA has been invoked as a positive regulator of initiation of DNA replication. DNA replication in *E. coli* starts at a fixed origin sequence on the chromosome (*oriC*) and progresses bidirectionally toward the terminus *terC* (**Figure 4**). *OriC* is a 258-bp sequence that is flanked by other regions required for the onset of DNA replication. Of particular importance are the so-called DnaA boxes that recognize the DnaA protein. DnaA becomes active when it binds ATP. When sufficient DnaA-ATP is available for occupation of the DnaA boxes, melting of the DNA double strands of the origin takes place, allowing replication proteins to bind. There are additional DnaA boxes on the chromosme but their function is not known. Conceivably, these boxes titrate away excess DnaA to prevent premature reinitiation of *oriC*. Continued protein synthesis serves to provide new DnaA molecules for a next round of replication.

An additional mechanism has been invoked that prevents premature initiation of DNA replication. This is based on the fact that newly synthesized DNA is hemimethylated in contrast to mature DNA, which is fully methylated. Hemimethylated DNA containing GATC sites becomes sequestered by a protein appropriately called SeqA, thus preventing re-initiation of DNA replication. The duration of the re-replication block is of the order of one third of Td. Presumably, this period in combination with a limited amount of DnaA is sufficient to prevent re-initiation prematurely.

DNA Replication Cycle in Archaea

Bacteria and archaea, though prokaryotes, differ considerably from one another with respect to DNA replication and possibly DNA segregation. This supports the increasing evidence that archaea are closer to eukaryotes than to bacteria. Though a limited number of archaea have been investigated some interesting features have emerged. For instance, in hyperthermophile, *Sulfolobus* spp., which belong to the archaeal phylum Crenarchaeota the chromosome contains three different origins. These initiate simultaneously, though termination appears variable in time. This is reminiscent of the multiple origins in a eukaryotic chromosome. In *S. solfataricus* pairing of replicating as well as postreplicating chromatids have been observed. This suggests the presence of genuine G2-period in this organism. Whether chromosome compaction precedes DNA segregation remains to be seen. However, it seems likely that DNA replication and DNA segregation do not run in parallel as in *E. coli*.

Division Cycle

The fastest division cycle in *E. coli* in rich medium at 37 °C is about 20 min, which is the same as the minimal D-period. Consequently, cells growing with a Td of 20 min are continuously involved in cell division. As outlined above such cells initiate DNA replication every 20 min, which makes multifork replication a must. The tight coordination between DNA replication and cell division (**Figure 3**) requires that cell division takes place at the right time and at the right place. How is this achieved?

The Divisome and Divisome Subassemblies

Most cell division proteins have been discovered through the phenotypes of temperature-sensitive cell division mutants. At the nonpermissive temperature cells grow as filaments, thus revealing defects in the division process. Many cell division proteins have the prefix Fts, meaning filamentation-thermosensitive. Genetic studies have been complemented by microscopic labeling studies, both by electron microscopical immunogold labeling (**Figure 6**) and fluorescent light microscopy (**Figure 7**). Cytokinesis is carried out by a protein machine called the divisome, which contains about 20 different proteins. The majority of them are specific for the divisome; others are also active during cell elongation. These proteins are located in different cellular compartments and are cytoplasmic proteins, integral transmembrane proteins, or periplasmic proteins with a membrane anchor. Recently, a protein complex has been identified (Tol-Pal), which connects the divisome to the outer membrane.

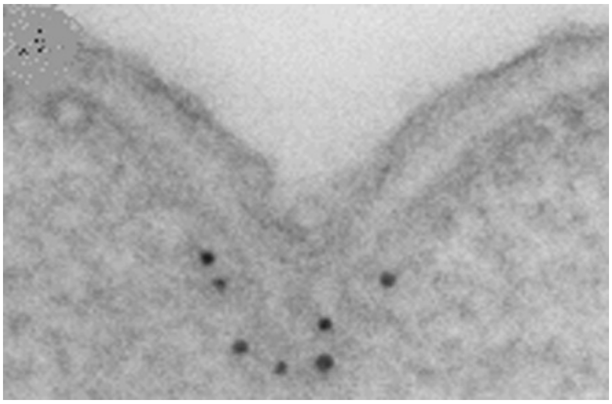

Figure 6 Electron microscopic image of immunogold labeled FtsZ in a thin section. Reproduced from Nanninga N (1998) Morphogenesis of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* 62: 110–129.

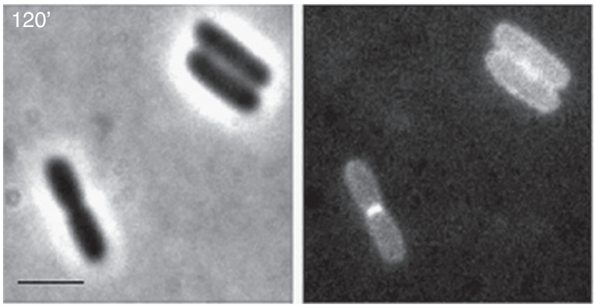

Figure 7 Fluorescent image (right) of FtsQ-GFP labeled live *Escherichia coli*. Left: phase contrast image. See attached film clip GFP-FtsQ. Courtesy of Dr. T den Blaauwen.

The first protein to arrive at the site of division is the cytoplasmic protein FtsZ (**Figure 6**). It is a tubulin homologue with GTPase activity. FtsZ occurs in thousands of copies per cell. Potentially, FtsZ forms one or more ring-like polymers underneath the cytoplasmic membrane in the cell center. The exact *in vivo* conformation of the FtsZ polymer(s) is (are) not known. It clearly is a dynamic structure, as demonstrated by photobleaching experiments. The Z ring must decrease in circumference during the constriction process, without losing its integrity. Whereas FtsZ occurs in thousands of copies in a cell, other proteins like FtsQ (**Figure 7**) are present in only about 50 copies per cell. Thus, such proteins cannot participate in forming a ring that spans the circumference of the cell. Presumably, FtsQ and other low-copy number proteins are grouped into divisome subassemblies, which decorate extended FtsZ polymers (**Figure 8**).

Recent investigations have revealed the temporal sequence of divisome biogenesis. The assembly of a functional divisome takes place in two steps. In the first step, the cell division proteins FtsA, ZipA, and ZapA bind and stabilize an initial FtsZ ring. In the later stage, the other cell division proteins are recruited, including FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI (PBP3; penicillin-binding protein 3), FtsN, and AmiC. Presumably, a subassembly is composed of these proteins (**Figure 9**). Remarkably, a complex of FtsB, C, and L can exist outside the divisome. The function of the various proteins is largely unknown. FtsA is a member of the actin superfamily, FtsK has a role in separating chromosomes after termination of DNA replication, FtsI is a transpeptidase, and AmiC an amidase. The latter two proteins emphasize the importance of peptidoglycan synthesis during division.

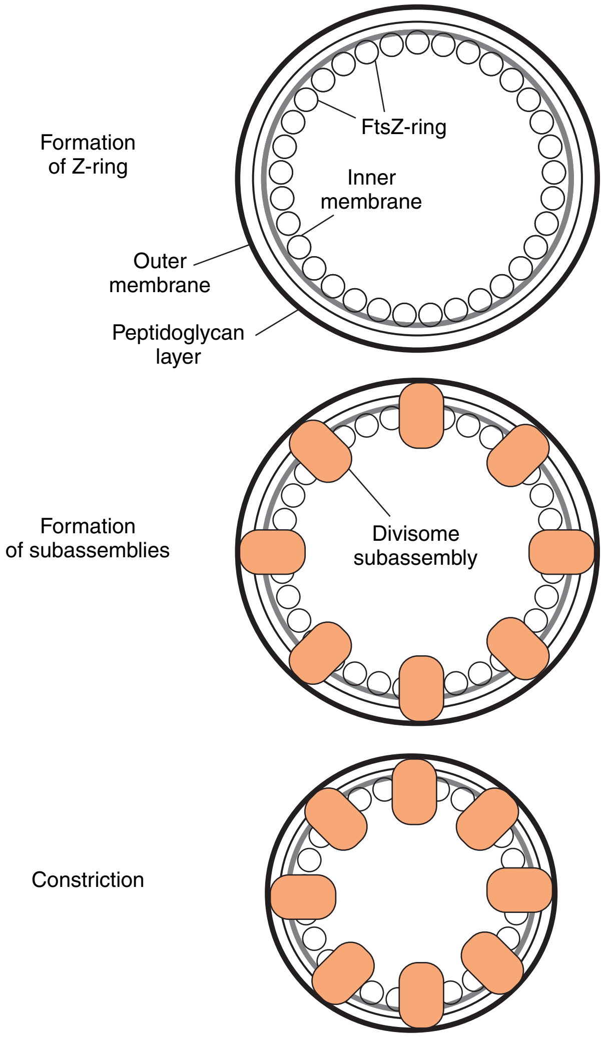

Figure 8 The division process in *Escherichia coli*. First the FtsZ ring with associated proteins (FtsA, ZapA, and ZipA; not shown) is formed. Next divisome subassemblies are positioned. During constriction the subassemblies approach each other, whereas FtsZ leaves the ring. Note that the various components have not been drawn to scale.

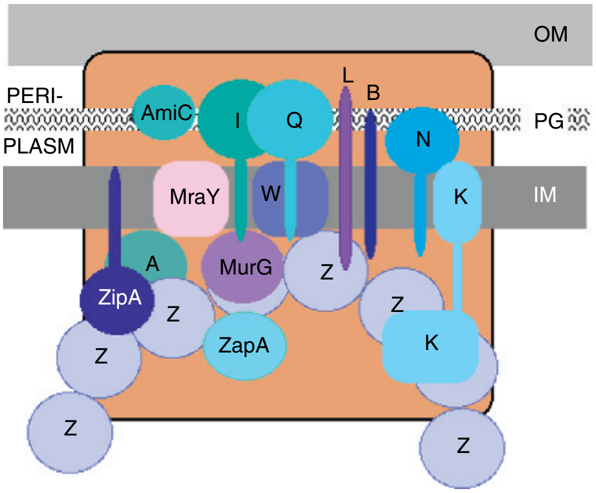

Figure 9 Protein composition of a divisome subassembly. The divisome subassembly spans cytoplasm, inner membrane (IM), peptidoglycan layer (PG), periplasm, and outer membrane (OM). It interacts with an FtsZ scaffold, which is reinforced by the cytoplasmic proteins FtsA, ZapA, and ZipA. The latter is anchored to the inner membrane. Specific components of the divisome subassembly are FtsB, FtsI, FtsK, FtsL, FtsN, FtsQ, and FtsW. Most of the proteins have their main domain in the periplasm as depicted. Note that FtsW is membrane-embedded. The cytoplasmic domain of FtsK functions in DNA segregation. Tol-Pal (not shown) connects the subassembly to the outer membrane.

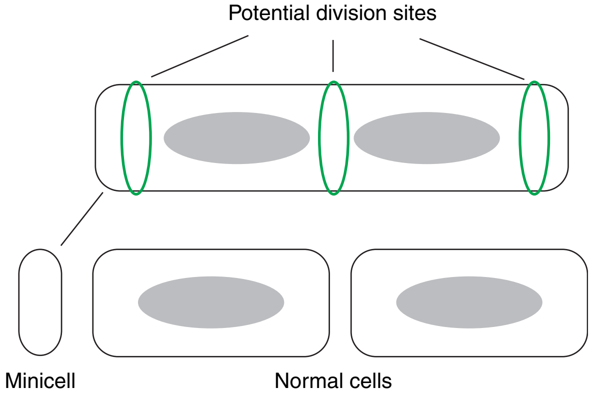

Figure 10 Potential division sites in a rod-shaped cell. Division takes place where there is no physical obstruction of the nucleoid, that is at poles and in the cell center. Normally division only occurs in the cell center. However, when the Min system is impaired a minicell is formed at a pole. Division starts by the positioning of a Z ring (green) composed of polymerized FtsZ.

Potential Division Sites and Site Selection

Although it is customary to think that cells split in the middle, additional potential division sites exist at the cell poles (**Figure 10**). As depicted here, division takes place at regions that are not near the nucleoid (nucleoid occlusion; see 'Cell cycle regulation'). Interestingly, polar divisions occur in thermosensitive *min* mutants and result in the so-called minicells. These are devoid of DNA, though not of ribosomes, thus can still carry out some residual protein synthesis. The phenomenon of minicell production indicates the existence of a system to prevent polar divisions. It is known as the Min system. The protein MinC inhibits polymerization of FtsZ at the cell poles. MinC is recruited to the membrane by the ATPase MinD in its ATP-bound form. Binding of MinD to the membrane is released by interaction with MinE, which causes ATP hydrolysis. MinE is specifically active at the cell center, thus preventing inhibition of FtsZ polymerization by MinC. Remarkably, the polar topology of these reactions is achieved by oscillation of MinD from pole to pole. The time course of one oscillation is of the order of 1 min. The Min system, as described for *E. coli*, is not found in *B. subtilis* and *C. crescentus*. Interestingly, Min proteins (and FtsZ) have been found in chloroplasts and in some mitochondria, which emphasizes their endosymbiontic origin of the latter.

Though the mechanism of division site selection is not fully understood, elements include the sensing of the cellular position of the nucleoid (see below) and the inhibition of polar divisions by the Min system.

Peptidoglycan Synthesis during Cell Elongation

After birth, rod-shaped cells like *E. coli* elongate before they divide. This implies growth of the covalent peptidoglycan layer and insertion of new components in the noncovalent inner and outer membrane. Precursors of building blocks for the peptidoglycan layer are produced stepwise in the cytoplasm. The final cytoplasmic products are lipids I and II, which are made by the enzymes MraY and MurG, respectively. MraY, a translocase, is an integral membrane protein that binds UDP-MurNAc-pentapeptide to undecaprenyl phosphate forming lipid I. MurG, a transferase, is tightly associated with the cytoplasmic side of the inner membrane. It adds UDP-GlcNAc to lipid I producing lipid II. An unknown flippase activity transfers the disaccharide moiety of lipid II to the periplasmic side of the inner membrane, where it serves as a substrate for penicillin-binding proteins. Elongation of the glycan chains of existing peptidoglycan is carried out by a transglycosylase activity and cross-linking of peptide side chains is due to a transpeptidase activity. Some PBPs, such as PBP1a and PBP1b, are bifunctional and perform both activities. PBP2 is monofunctional with only a transpeptidase activity. PBP2 is essential for the cells' rod shape, because mutations or binding by the PBP2-specific antibiotic mecillinam turns rods into spheres. Light microscopic fluorescent studies have shown that PBP1b and PBP2 are dispersed along the lateral wall, with additional label at the site of constriction (see also below). Another rod-shape determinant is RodA, a transmembrane protein, whose biochemical function is not yet known.

In recent years, increasing evidence points to the presence of macromolecular complexes that connect cytoplasm and periplasm to carry out peptidoglycan synthesis at many site along the cells' length. These complexes do not seem to be randomly located, but are probably arranged in a helical fashion, with the actin-like MreB polymer serving as a scaffold (**Figure 11**). Such an arrangement can explain why a diffuse incorporation of peptidoglycan precursors has been found in earlier studies. Earlier studies have also shown that outer membrane assembly is a random process along the cell length, presumably also obscuring the helical insertion of its components.

Though we have focused on PBPs with synthetic activities, there are numerous other enzymatic activities that lead to remodeling and recycling of peptidoglycan. In this sense, **Figure 11** represents only a first approximation of the *in vivo* situation.

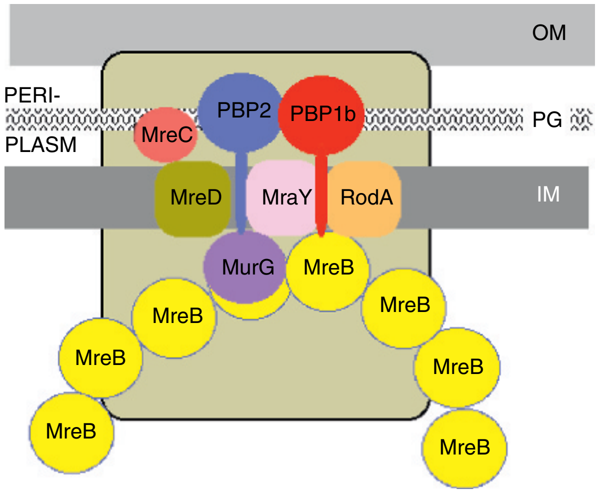

Figure 11 Protein machine involved in cell elongation. It encompasses components in cytoplasm, inner membrane, and nascent peptidoglycan. IM, inner membrane; OM, outer membrane; PG, peptidoglycan layer. The peptidoglycan synthesizing machinery including MurG, MraY, PBP1b, and PBP2 is positioned by a helical MreB polymer underneath the inner membrane. This model integrates data from *Escherichia coli* and *Caulobacter crescentus*.

Peptidoglycan Synthesis at the Divisome

Constriction is not simply the pulling inward of the envelope by an FtsZ ring of a decreasing circumference. The constriction process requires local envelope synthesis at least on the level of the peptidoglycan layer. Early electron microscopic autoradiographic studies have shown that the peptidoglycan precursor [^{3}H]-*meso*-diaminopimelic acid is especially incorporated at the site of constriction. FtsI (PBP3) is involved in divisome-specific peptidoglycan synthesis. It is the most intensively studied of all cell division proteins. Certain antibiotics, such as cephalexin, furazlocillin, and aztreonam, act on this protein specifically. Inhibition of FtsI by these antibiotics or inactivation of FtsI in a thermosensitive *ftsI* mutant at the nonpermissive temperature produces filaments with aborted blunt constrictions. This is in contrast to filaments of thermosensitive *ftsZ* mutants, which have a smooth morphology. Peptidoglycan assembly does not take place at the blunt constrictions. Remarkably, inhibition of FtsI does not prevent peptidoglycan synthesis at new division sites flanking the blunt constrictions (**Figure 12**). This has been demonstrated by electron microscopic autoradiography and by viewing the dilution of immunogold label attached to –SH groups of D-Cys incorporated into the peptidoglycan layer. It is plausible that initial peptidoglycan synthesis at

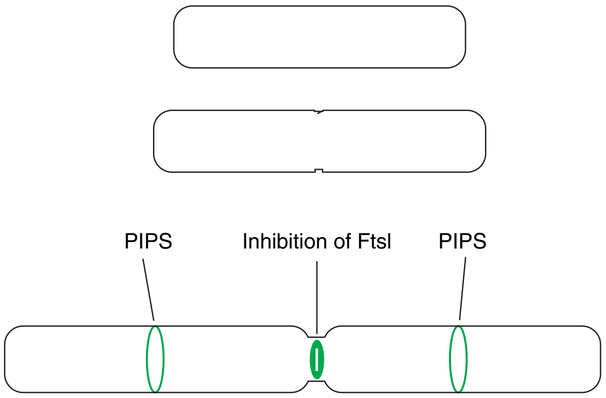
PIPS
Inhibition of FtsI
PIPS

Figure 12 Inactivation of FtsI (PBP3) halts constriction and produces blunt constrictions. Incipient division remains possible at future division sites flanking the aborted constriction. FtsZ rings (green) form and PBP3-independent peptidoglycan synthesis (PIPS) proceeds.

the division site is carried out by PBP2 and PBP1b as remnants of the cell elongation system. The phenotype of FtsI-impaired cells has led to an early suggestion that FtsI plays a role in a later stage of cell division. It also fits the idea that FtsI becomes recruited to the assembling divisome in a second step (see above).

The localized function of FtsI requires the presence of enzymes, which prepare the substrate prenylated disaccharide peptide (lipidII) for PBPs to act upon in the periplasm. During cell elongation these enzymes are MraY and MurG. Most likely, these proteins are also present in the divisome (**Figure 9**).

Organization of Cell Division Genes on the Chromosome

The *E. coli* chromosomal map is divided into 100 min. In this organism most cell division genes are located at 2 min. Remarkably, many genes encoding enzymes involved in the formation of peptidoglycan precursors and in peptidoglycan assembly are also situated in this region. The 2-min region is therefore also denoted as the *dcw* cluster, where *d* stands for division and *cw* for cell wall (**Figure 13**). This clustering suggests the existence of a global regulatory mechanism that coordinates expression of the many genes and which directs the transformation of elongation-specific to division-specific peptidoglycan synthesis. To date, little is known about division-specific gene expression. Recent studies on genomic sequences have shown that the *dcw* cluster is conserved among bacteria (**Figure 8**). Note that archaea have no peptidoglycan and, consequently, no PBPs.

Cell Cycle Regulation

In the *E. coli* cell cycle the frequency of initiation of DNA replication equals Td. If $C + D < Td$, newborn cells contain a replicating chromosome, which exemplifies the fact that a newborn cell is not completely new. If $C > Td$, multifork replication ensues. Cells have to know when to initiate DNA replication and when to start cell division. Though some molecular aspects have been discussed above, a complete picture is still far off. Transcriptional regulation through a central controller might be a possible means to exert control on cell cycle events. However, cellular biochemical processes might be interwoven in such a way that the concept of a central controller might not apply.

It is intriguing that cells are capable to adjust for DNA damage via the so-called SOS response (see below). Another regulating aspect resides in the cells' perception of the location of its nucleoid, thus prohibiting the cutting of its DNA by the constricting envelope (nucleoid occlusion).

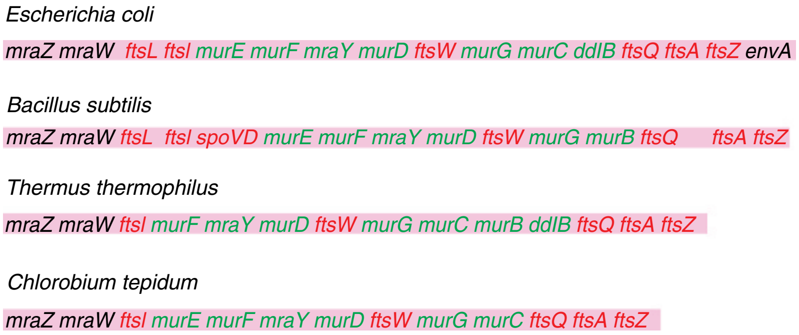
Escherichia coli
mraZ mraW ftsL ftsI murE murF mraY murD ftsW murG murC ddlB ftsQ ftsA ftsZ envA
Bacillus subtilis
mraZ mraW ftsL ftsI spoVD murE murF mraY murD ftsW murG murB ftsQ ftsA ftsZ
Thermus thermophilus
mraZ mraW ftsI murF mraY murD ftsW murG murC murB ddlB ftsQ ftsA ftsZ
Chlorobium tepidum
mraZ mraW ftsI murE murF mraY murD ftsW murG murC ftsQ ftsA ftsZ

Figure 13 Genes involved in cell division (*d*) and cell wall (*cw*) synthesis are grouped together (*dcw* cluster) in a wide range of bacteria. In *Escherichia coli* they are located at the 2-min region of the chromosome. Genes encoding for cell division proteins are shown in red; those involved in cell wall synthesis in green. Reproduced from Mingorance J, Tamames J, and Vicente M (2004) Genomic chanelling in bacterial cell division. *Journal of Molecular Recognition* 17: 481–487.

SOS- Response

DNA damage prevents completion of DNA replication and segregation. As a consequence cell division is inhibited. Damage can occur, for instance, upon UV radiation or by treating cells with a chemical like nalidixic acid. Cell division is postponed until damaged DNA has been repaired (if possible). This safeguarding of cell division until DNA has been properly repaired is called the SOS response. If damage leads to the formation of single-stranded DNA (ssDNA) at a replication fork, a protein called RecA polymerizes on the ssDNA. This in turn depletes a repressor called LexA, which allows the expression of the cell division inhibitor SulA (SfiA), among other things. SulA inhibits the polymerization of FtsZ at the cytoplasmic membrane in the cell center.

Nucleoid Occlusion

Potential division sites (**Figure 10**) exist where there is no spatial obstruction by the nucleoid. This implies that the divisome is assembled after nucleoids have segregated upon completion of DNA replication. This is known as the nucleoid occlusion model. So far little is known about the sensing mechanism that links termination of DNA replication (and segregation) with initiation of division. However, recently DNA-binding proteins (Noc and SlmA), which seems to interfere with divisome assembly, have been detected in *B. subtilis* and *E. coli*, respectively.

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Cell Membrane, Prokaryotic

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Defining Statement
Introduction
Membrane Fluidity
Bacterial CMs
Gram-Negative Bacterial OMs
OMs of Acid-Fast Gram-Positive Bacteria
Archaeal Membranes
Conclusions
Further Reading

Glossary

archaea One of the three domains of living organisms: archaea, bacteria, and eukarya. Although archaea share a basic morphology with bacteria and they are also prokaryotes (i.e., they lack a true nucleus), in many molecular details they resemble eukaryotes more than bacteria. Previously called archaebacteria.

cell membrane A phospholipid bilayer that surrounds all cells. Also called cytoplasmic membrane or plasma membrane.

cell wall The tough envelope surrounding many cells, including nearly all bacteria and archaea. Located outside the cytoplasmic membrane.

channel proteins Proteins that form aqueous pores or channels through membranes.

crystalline surface layer A surface layer (S-layer) of some bacteria and archaea consisting of protein arrays, usually quite resistant to chemicals and proteases.

domain (1) A discrete, independently folded region of a protein. Different subfunctions of a multidomain protein are usually localized in separate domains. (2) One of the three major taxons: bacteria, archaea, or eukarya.

electron transport chain The sequential oxidation/reduction of compounds embedded in a membrane that creates proton/sodium gradients across membranes.

facilitated diffusion Movement of molecules across a membrane from higher to lower concentration mediated by proteins that permit the passage of specific molecules only.

glycerol ether lipid A type of lipid, characteristic of the archaea, containing isoprenoid lipids that are ether linked to glycerol.

Gram-negative bacteria A group of bacteria with cell envelopes composed of two membranes, the inner and the outer, lipopolysaccharide-containing membranes as well as thin peptidoglycan cell wall layers.

Gram-positive bacteria Two groups of bacteria, both with thick peptidoglycan cell layers. One group, the low G+C Gram-positive bacteria, lacks an outer membrane. The other, the high G+C Gram-positive 'acid-fast' bacteria, has a thick outer cell membrane overlying the cell wall.

lipid A A phosphorylated glycolipid common to all Gram-negative bacterial lipopolysaccharides.

lipopolysaccharides Major components of the outer monolayers of the outer membranes of most Gram-negative bacteria. Abbreviated LPS.

lipoprotein A protein containing covalently bound fatty acids.

mycolic acid A long-chain organic acid found in the waxy cell envelope of mycobacteria and related acid-fast high G+C Gram-positive bacteria.

outer membrane The outer lipid bilayer of many prokaryotes and some eukaryotic organelles. In Gram-negative bacteria, they consist of an outer lipopolysaccharide leaflet and an inner phospholipid leaflet plus proteins. In high G+C Gram-positive bacteria, the outer membranes incorporate mycolic acids.

passive transport Diffusional passage of a compound across a membrane.

permease A proteinaceous system functioning in the transport of specific substances through a membrane.

phospholipid bilayer A membrane consisting of two leaflets, each composed of phospholipid.

proton motive force (PMF) Potential energy stored in the form of an electrochemical gradient of protons across a cell or organellar membrane.

secondary active transport Active transport of substances using the sodium or proton motive force as the energy source driving substrate accumulation or efflux.

sodium motive force (SMF) Potential energy stored in the form of an electrochemical gradient of sodium ions across a cell or organellar membrane.

symbiosis The living together of two different kinds of organism in a nonharmful fashion.

symport The coupled movement of two molecules together across a cell membrane. Usually, the concentration gradient of one of them drives the movement of the other.

Abbreviations

ATP	adenosine triphosphate
CM	cytoplasmic membrane
DMSO	dimethyl sulfoxide
GTP	guanosine triphosphate
KDO	keto deoxy octulosonate
LPS	lipopolysaccharides
OM	outer membrane
OMP	outer membrane protein
PEP	phosphoenolpyruvate
PIM	phosphatidylinositol mannoside
PMF	proton motive force
SAM	sorting and assembly machinery
SMF	sodium motive force
Tat	twin arginine targeting/translocating
TCDB	Transporter Classification Database
TMAO	trimethylamine *N*-oxide
TMS	transmembrane segment
UDP-GlcNAc	uridyl-diphospho-*N*-acetylglucosamine

Defining Statement

In addition to cytoplasmic membranes, shared by all living cells, many prokaryotes possess protective outer membranes that have compositions very different from those of their inner membranes as well as from those of the outer membranes of other organismal types. This chapter describes these membranes.

Introduction

Bacterial cytoplasmic membranes (CMs) consist primarily of amphipathic phospholipids with lesser amounts of glycolipids. In the case of Gram-negative bacterial outer membranes (OMs), phospholipids are present in the inner leaflet of the bilayer while lipopolysaccharides (LPS) predominate in the outer leaflet. Most Gram-positive bacteria lack an OM. This is true of all low G+C Gram-positive bacteria, called ‘firmicutes’, although acid-fast, high G+C Gram-positive bacteria, such as *Mycobacterium* species, have mycolic acid-containing OMs that are structurally very different from the OMs of Gram-negative bacteria. Like Gram-positive bacteria, most archaea lack an OM, but recent studies have revealed the occurrence of archaea with these structures. The lipid constituents of both the inner and OMs of archaea are very different from those of bacteria. The OMs of all of these organisms provide a degree of protection from toxic substances, not found in organisms having a single membrane.

Almost all bacterial membranes are assembled in bilayers with embedded integral and associated peripheral membrane proteins. Lesser amounts of carbohydrates (glycolipids and glycoproteins) extend outward from these membranes. Properties of bacterial membrane proteins and lipid–protein interactions have been studied in detail as have those of archaeal membranes. The latter unique membranes often consist of hydrophobic tails linked by ether rather than ester bonds to the glycerol-containing lipid backbone. A few archaea have complex envelopes which, like those in some bacteria, consist of inner and outer membranes that are of different lipid and protein contents.

Our understanding of prokaryotic cell membrane dynamics has advanced considerably since the fluid mosaic model was proposed by Singer and Nicolson in 1972. Moreover, links between CM structure and function have been extensively elucidated. However, our understanding of prokaryotic membranes remains incomplete. For example, we do not yet fully understand what aspects of membrane physiology are responsible for bacterial or archaeal survival in diverse and extreme environments.

Lipid and protein membrane compositions are central to survival since prokaryotes in general are subject to extreme physical and chemical stresses. The CM should be thought of as the primary boundary between the external environment and the living cell while the OM serves a primary protective function. Flexibility in the adaptive capacity of the envelope and its components to environmental conditions is a primary determinant of cell survival.

The selectively permeable envelope allows appreciable diffusion of small neutral molecules such as H_2O, NH_3, CO_2, and O_2, although transport proteins may increase diffusional rates or allow accumulation against concentration gradients. However, this envelope presents high-energetic barriers to the permeability of moderately sized and large polar molecules. The CM serves the cell in numerous capacities providing functions including active transport, macromolecular synthesis, energy generation, maintenance of electrochemical gradients, and cell division.

Membrane Fluidity

Microbial membranes provide a fluid matrix for embedded proteins. The membrane state is frequently defined in terms of degree of fluidity. Fluidity reflects the lipid order and microviscosity which in turn are determined by lipid shape and packing. A composite measure of the lateral and rotational movement of lipids seems to determine membrane lipid/protein phase behavior.

Static as well as dynamic properties are characteristic of all biological membranes. Changes in CM fluidity may reflect physical and chemical interactions with environmental factors such as temperature, pH, osmotic pressure, internal and external ion compositions, and the presence or absence of various chemicals. Membrane perturbations elicit adaptive responses that must compensate for suboptimal conditions. Membrane alterations represent only one type of response, but these membrane responses, required to maintain cell function, guarantee a degree of fluidity that allows transmembrane transport as well as lateral lipid and protein diffusion without jeopardizing membrane stability.

Lipids exhibit polymorphism; each lipid has a distinctive headgroup and two dissimilar fatty acids. They aggregate into different structures such as bilayers and micelles, and assume structurally distinct phases. These depend on forces including exclusion volume, headgroup interactions, and van der Waals interactions between hydrocarbon chains. Lipid behavior within bacterial membranes is complicated by the huge variety of lipid types present. This diversity makes lipids among the most flexible and versatile of the various types of biological macromolecules.

The dynamic phase behavior of membranes balances the gel-to-liquid crystalline and the lamellar-to-nonbilayer phase transitions. Cumulative evidence suggests that mechanisms of cellular homeostasis are integrated elements of the dynamic range of membrane behavior. Methods used to study fluidity include X-ray diffraction, differential scanning calorimetry, electron spin resonance, nuclear magnetic resonance using ^{2}H or ^{31}P, and fluorescence polarization. Visualization of the membrane has been achieved by electron microscopy and X-ray diffraction. Fluorescence polarization and several of the other cited methods can be used to measure the static versus dynamic characteristics of the membrane. The first of these techniques has the advantage that it can be used with viable bacteria under a variety of normal and stressed conditions as well as *in vitro* with isolated membrane preparations.

Molecular studies have revealed the types of lipid order and phase alterations employed by prokaryotes to maintain CM fluidity and function. The use of extracted bacterial and archaeal lipids has revealed differences of *in vitro* versus *in vivo* properties resulting from membrane adaptations. CM polarization data for bacteria have been obtained under normal and a variety of environmental stress conditions, revealing the responses of membranes to these conditions.

Bacterial CMs

The CM of any bacterium is an ~80-Å-thick structure separating the interior of the cell from the environment. It prevents the diffusion of most substances into and out of the cytoplasm and acts as a selective barrier to concentrate metabolites and nutrients within the cell while secreting waste products and toxins. These structures will be reviewed here while OMs of bacteria will be described in the sections titled 'Gram-negative bacterial OMs' and 'OMs of acid-fast Gram-positive bacteria'. Archaeal membranes will be discussed in the section Archaeal membranes.

Structure, Composition, and Function of CMs

The CMs of most bacteria consist of roughly equal amounts of phospholipid and protein (see **Figure 1(a)**). They contain ~70% of the cellular phospholipids and 25% of the cellular proteins. The phospholipids are amphipathic, having hydrophobic tails and hydrophilic heads. The glycerol backbone contains two bound fatty acids and a phosphoryl headgroup. Three major types of phospholipids are present in *Escherichia coli*, about 2×10^{7} molecules per cell: 75% phosphatidylethanolamine, 20% phosphatidylglycerol, and 5% cardiolipin (diphosphatidylglycerol). All of these phospholipids contain α-glycerol-phosphate esterified with fatty acids at the one and two positions. The predominant fatty acids in *E. coli* are palmitic acid (16:0), palmitoleic acid (16:1), and *cis*-vaccenic acid (18:1). Different bacteria have different ratios of these lipids, and many have others as well. Sterols, glycolipids, amino acid-containing lipids, and other hydrophobic and amphipathic molecules can be present, depending on the species.

The CM is stabilized by hydrophobic interactions and hydrogen bonds; the former are also known as van der Waals interactions. In addition, divalent cations such as Mg^{2+} and Ca^{2+} stabilize the membrane by neutralizing the negative charges of the phospholipids on both sides of the bilayer, serving as 'salt bridges'. The asymmetric bilayer, with different lipid and protein compositions for the two apposed monolayers, is thus a stable structure that serves as an encapsulating 'bubble' for the cell cytoplasm. It is absolutely essential for all living cells.

As noted above, the fatty acid composition of the phospholipids that comprise the CM (e.g., chain length, substitutions, and degree of saturation) are determined during biosynthesis, dependent on internal and environmental conditions such as stage of growth, temperature, and composition of the external milieu. The CM maintains a fluid state to allow conformational flexibility and lateral diffusion of proteins and protein complexes. Fluid membranes also have higher transmembrane

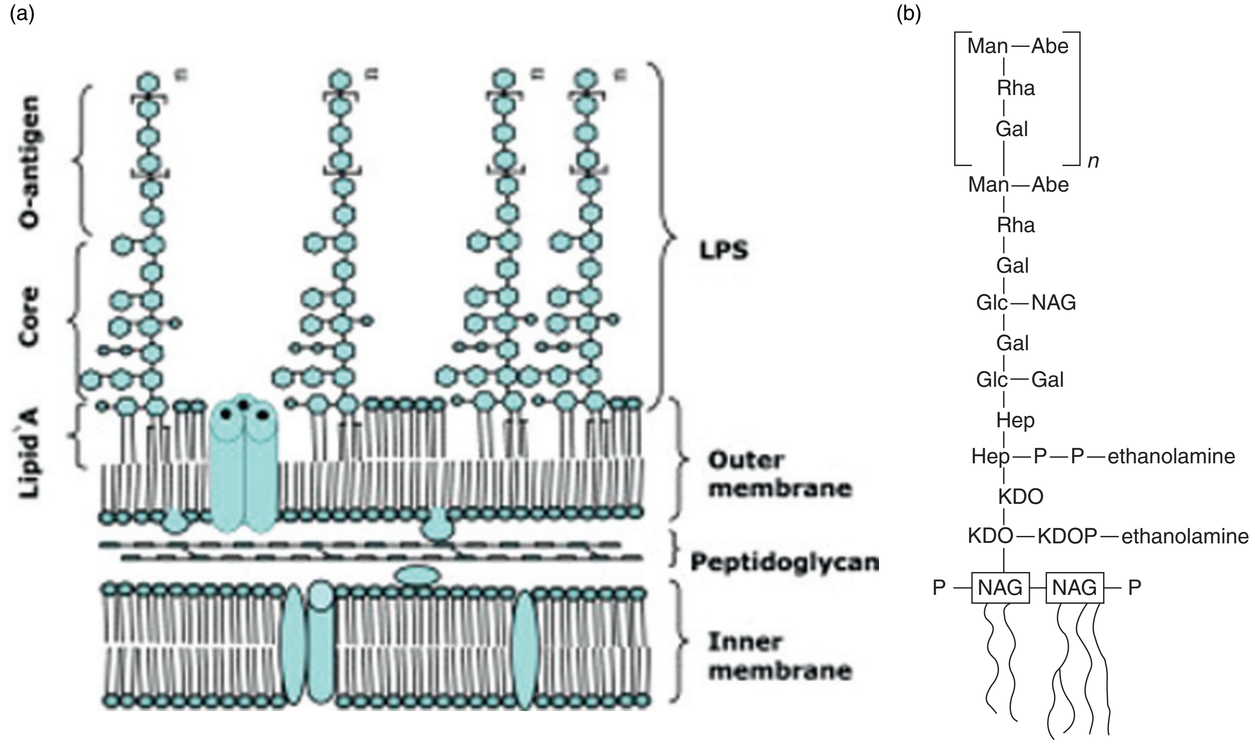

Figure 1 (a) Schematic view of the *Escherichia coli* cell envelope. Lipopolysaccharide (LPS), embedded within and extending from the outer surface of the outer membrane, consists of three moieties: lipid A, core polysaccharide, and the O-antigen polysaccharide side chains. A trimeric porin in the outer membrane and integral membrane proteins in the inner membrane are depicted schematically. The peptidoglycan cell wall in the periplasm separates the two membranes. (b) Structure of the *E. coli* lipopolysaccharide (LPS) showing the sugar residues. KDO, keto deoxy octulosonate; Hep, heptose; Glc, glucose; Gal, galactose; NAG, *N*-acetylglucosamine; Rha, rhamnose; Man, mannose; Abe, abequose; *n*, a variable number of repeat units.

permeabilities to small molecules than do more rigid bilayers. As noted above, the phospholipids occur primarily in a bilayer, forming a hydrophobic barrier, but micellar structures and 'lipid rafts' of unusual composition may be present, illustrating their dynamic nature. The membrane prevents the unregulated transmembrane movement of polar molecules and allows the selective retention of ions, essential metabolites, and macromolecules. Due to the presence of specific transport systems, it also catalyzes the active extrusion from the cell of end products of metabolism, drugs, and toxins.

Integral membrane proteins in the CM are anchored into the membrane with one or more transmembrane segments (TMSs). Others, peripheral membrane proteins, are loosely bound and interact transiently, often due to ionic attractive forces. Analyses of the *E. coli* protein content (the proteome) indicate that about one quarter of the predicted gene products are integral membrane proteins in the CM. Many are critical for cellular functions (e.g., transport and cell division). The largest functional class is the transport proteins which comprise 5–15% of the total proteome, depending on the organism. Owing to their hydrophobic and amphiphilic characters, membrane proteins are much more difficult to study than soluble (cytoplasmic and extracellular) proteins. They account for less than 1% of the known high-resolution protein structures, solved by either X-ray crystallography or multidimensional nuclear magnetic resonance. Topological models have been derived that depict the number of TMSs and the orientation of the proteins in the lipid bilayer, and in numerous cases, these structural predictions have been verified experimentally. However, in only a few cases do we know how the various TMSs interact with each other to form compact, functional proteins and protein complexes.

Amino acid residues of the portions of proteins that are embedded in the membrane have hydrophobic character, as do residues in the cores of soluble proteins. Residues in the same membrane proteins that are exposed to the aqueous environment, however, are much more polar. Residues in membrane proteins that are exposed to lipid acyl side chains have greater hydrophobic character than do residues in the protein interior. The latter are often semipolar and are important for maintaining correct conformation of the lipid bilayer. In general, TMSs in the CM are hydrophobic α-helices.

In addition to transport, CM proteins can be involved in transmembrane electron flow, energy generation and conservation, biosynthesis of hydrophobic substances, synthesis of cell envelope constituents, and translocation of cell wall and envelope macromolecules from the inside of the cell to an extracytoplasmic locale. This last mentioned function can include translocation and insertion of proteins through and into the one or two membranes of the bacterial cell envelope. Thus, there are at least five compartments in the Gram-negative bacterial cell: the cytoplasm, the inner membrane, the periplasm between the two membranes, the OM, and the extracellular milieu. Moreover, in both membranes, the inner and outer leaflets of these bilayers can be considered as distinct compartments. Specific, dissimilar, and evolutionarily distinct protein insertion complexes are responsible for integration of inner and outer membrane proteins (OMPs) into the envelope. It is also important to note that certain bacteria have been shown to possess a variety of membrane-bounded organelles such as magnetosomes which allow bacteria to orient in the Earth's magnetic field, chromatophores where photosynthesis occurs, gas vacuoles that provide the function of flotation, and sulfur granuoles that house elemental sulfur. The complexity of the prokaryotic cell is thus far greater than was initially believed.

Energy Generation and Conservation

Many biosynthetic and CM transport processes are driven by the hydrolysis or transfer of the high-energy phosphoryl moieties of adenosine triphosphate (ATP), guanosine triphosphate (GTP) or phosphoenolpyruvate (PEP). In many cases, the phosphoryl group is transferred transiently to a protein or solute, whereas in a few other cases, phosphoryl bond hydrolysis drives the formation of high-energy protein conformational states; yet other transport processes are energized by transmembrane ion gradients. Cells growing under fermentative conditions, in the absence of oxygen or another inorganic electron acceptor, produce ATP by substrate-level phosphorylation reactions as in the glycolytic pathway. Generally, bacteria that generate energy primarily by substrate-level phosphorylation (fermentation) use ATP or PEP to drive the majority of their transport processes, whereas bacteria that generate energy primarily via electron transfer (respiration) use ion (proton and sodium) gradients to drive most of their transport processes. In the former case, the ATP synthesized by substrate-level phosphorylation can also be used to form transmembrane ion gradients by using the F_1F_0 proton-translocating ATPase, while ion gradients generated via respiration can be used to synthesize ATP in the reverse process catalyzed by the same enzyme complex. Marine bacteria more frequently use Na^+ gradients to drive transport than observed for fresh water or terrestrial bacteria.

For cells growing under respiratory conditions, the passage of electrons through an electron transfer chain to suitable electron acceptors (oxygen, fumarate, nitrate, nitrite, dimethyl sulfoxide (DMSO), trimethylamine *N*-oxide (TMAO) or hydrogen, e.g.) can be coupled to the extrusion of protons or sodium ions and the creation of transmembrane electrochemical gradients. The resultant proton motive force (PMF, $\Delta\mu_H$) or sodium motive force (SMF, $\Delta\mu_{Na^+}$) is essential for life and can be used to drive transport. However, the transmembrane electrochemical gradient is also important in maintaining protein conformations, opening channels, and influencing transmembrane enzyme activities.

Bacterial respiratory chains consist of a series of physically separate protein complexes. Commonly, membrane-bound dehydrogenases transfer two electrons and/or hydrogen atoms from their substrates to the pool of quinones. Electron donors in bacteria include reduced molecules such as NADH, succinate, α-glycerol phosphate, nitrite, and sulfides. Quinones serve as mobile hydride carriers diffusing through the membrane. These quinones shuttle reducing equivalents from the dehydrogenases to terminal reductases or oxidases that oxidize the electron acceptors (e.g., oxygen, nitrate, and H_2) listed in the preceding paragraph. While ubiquinone-8 is the predominant quinone species in aerobically grown *E. coli* cells, menaquinone-8 is the major species in cells grown anaerobically. Several, but not all of these respiratory/electron transfer complexes, catalyze proton or sodium ion export during electron flow. This proves to be one of the primary mechanisms for generating ion motive forces (the PMF and SMF).

Translocation of Proteins

Integral CM proteins need to be integrated into the membrane, and hydrophilic proteins need to be translocated through the CM from the inside of the cell where they are made, to the external cell surface where they function. For these purposes, the general secretory (Sec) pathway and the twin arginine targeting/translocating (Tat) pathway which act on unfolded and folded proteins, respectively, are usually used. A protein, YidC, assists insertion of proteins into the CM, alone or in conjunction with the Sec pathway. Especially in Gram-negative bacteria, but also in other prokaryotes, other protein complexes are integrated into the CM, working in conjunction with outer-membrane complexes destined to secrete proteins into the medium. Altogether, 16 distinct protein insertion/secretion systems have been identified in Gram-negative bacteria: eight each for transport across or into the inner and outer membranes, respectively. Some of these systems translocate their protein substrates across the two membranes in a single energy-coupled step.

Solute Transport

Nonpolar substances such as fatty acids, neutral alcohols, and simple aromatic compounds enter and exit the cell to some extent by dissolving in the lipid bilayer. By contrast, charged molecules such as organic acids and inorganic salts must be specifically transported. Water penetrates the membrane fairly freely, being small and uncharged, but aquaporins may facilitate the process, allowing more rapid water fluxes than would otherwise be possible in response to osmotic stress conditions. Similarly, passage of NH_3 and CO_2 through the membrane can be stimulated by the presence of 'gas channels'. More polar molecules are transported via specific membrane transporters, but there are many different types and hundreds of families of these proteins as tabulated in the Transporter Classification Database (TCDB). Active transport mechanisms allow the accumulation and extrusion of solutes against concentration gradients, while facilitated diffusion merely allows the energy-independent equilibration of the substrate across a membrane.

Most solutes are transported across prokaryotic CMs by energy-dependent mechanisms, and prokaryotes possess a remarkable array of active transport systems. These systems usually exhibit high substrate affinity and stereospecificity; the affinities reflect the concentrations of the solutes in the natural environments of these organisms. Mechanisms of energy-coupling to transport include: symport with and/or antiport against ions, ATP or GTP hydrolysis, phosphoryl transfer from phosphoenolpyruvate to sugar substrates (group translocation), organic acid decarboxylation, methyl transfer, light absorption, and electron flow. Group translocation involves the simultaneous transport and modification of substrates, often involving the expenditure of phosphoryl bond-type energy.

Gram-Negative Bacterial OMs

Gram-negative bacteria, and some Gram-positive bacteria, and archaea are surrounded by OMs which serve as selective permeation barriers. They prevent the entry of noxious compounds while allowing the influx of nutrients. They contain β-structured porins and other proteins that allow selective permeability and catalyze specific reactions. These membranes in Gram-negative bacteria will be considered in this section. Those in Gram-positive bacteria will be discussed in the section titled 'OMs of acid-fast Gram-positive bacteria', and those in archaea will be considered in the section titled 'Archaeal membranes'.

Structure and Composition of the OM

The OMs of most Gram-negative bacteria are asymmetric lipid bilayers where the inner leaflet contains phospholipids while the outer leaflet contains a preponderance of LPS (**Figures 1(a)** and **1(b)**). Gram-negative bacteria lacking LPS may instead have sphingolipids and/or various glycolipids. These bilayers show low permeability to many solutes. Amino acids, most vitamins, short peptides, sugars, etc. can cross the OM by diffusion through porin channels if smaller in mass than 600 Da. These channel proteins form β-barrel structures with transmembrane spanning segments consisting of amphipathic antiparallel β-strands. α-Helical proteins in the OMs of these organisms are rare, just as are β-structured proteins in the inner membranes. β-Barrel porins, in general, do not concentrate their substrate solutes across the membrane; they catalyze facilitated diffusion.

Other compounds such as vitamin B_{12} and iron siderophore complexes use substrate-specific, high-affinity active transporters to cross the OM. The energy required to allow these transporters to accumulate their substrates in the periplasm is derived from a complex of proteins (e.g., TonB, ExbB, and ExbD in *E. coli*) that use the PMF across the inner membrane to energize uptake. Since these receptors are exposed to the cell surface, infective agents such as some colicins and bacterial viruses (phage) can parasitize TonB-dependent systems or their homologues to enter and kill the host bacteria. Exactly how these protein complexes accumulate their substrates in the periplasm of the Gram-negative bacterial cell and allow passage of toxic proteins or DNA from phage into the cell is still an intense area of research.

The OMs of Gram-negative bacteria contain at least five major classes of proteins: (1) structural lipoproteins, (2) membrane-integrated β-barrel porins, (3) solute-specific receptors, (4) membrane-anchored enzymes, and (5) multicomponent surface structures such as fimbriae (organelles of adhesion), pili (organelles of conjugation), and flagella (organelles of motility). Lipoproteins usually have lipids and/or fatty acids covalently attached to an N-terminal cysteine. These tails, embedded in the OM, anchor these proteins within the membrane. β-Barrel proteins consist of β-sheets that are wrapped into cylinders. Many of these proteins (the porins referred to above) form channels allowing the free flow of nutrients and waste products. Porins can be nonspecific or specific for a particular class of substrates. Nonspecific porins act as 'molecular sieves', but the more specific porins may restrict permeation to a class of sugars, amino acids, ions, or other nutrient types. β-Barrel proteins may also possess enzymatic activities such as hydrolase activities. Moreover, as mentioned in the preceding paragraph, receptor proteins, embedded in the OMs via β-barrel structures, can accumulate their solutes in the periplasm against considerable concentration gradients using the PMF across the CM to drive uptake.

OM Lipopolysaccharides

LPSs (**Figures 1(a)** and **1(b)**) are found uniquely in most Gram-negative bacterial OMs. They are composed of three parts: the proximal, hydrophobic lipid A region which is embedded in the outer leaflet of the OM; the distal, hydrophilic O-antigen polysaccharide region that protrudes into the medium; and the core oligosaccharide region that connects lipid A to the O-antigen repeat units (**Figure 1(a)**). Lipid A is a polar lipid of unusual structure in which a backbone of glucosaminyl-β-$(1 \rightarrow 6)$-glucosamine is substituted with six or seven saturated fatty acyl residues.

In *E. coli*, LPS biosynthesis begins in the bacterial cytoplasm with the acylation of uridyl-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) with β-hydroxymyristate. After deacetylation, the product of this reaction is further modified with a second β-hydroxymyristate to generate UDP-2,3-diacylglucosamine. Cleavage of the pyrophosphate bond and displacement of the nucleotide, UMP, produces 2,3-diacylglucosamine-1 phosphate. After condensation of this compound with another molecule of UDP-2,3-diacylglucosamine and 4′ phosphorylation, the intermediate, lipid A, is formed. Two keto deoxy octulosonate (KDO; 3-deoxy-D-manno-oct-2-ulosonic acid) residues are then transferred, and two acyltransferases add lauroyl and myristoyl groups (**Figure 1(b)**). The core sugar residues are added onto this intermediate, and an export system translocates them from the cytoplasmic side to the periplasmic surface of the plasma membrane. The O-antigen, which is assembled and polymerized separately, is added in the periplasm, completing the biosynthetic process (**Figure 1(b)**). Subsequent transport reactions probably move the LPS molecules across the periplasmic space into the inner leaflet and finally to the outer leaflet of the OM (**Figure 1(a)**). Lipid A is the biologically active component of LPS which causes inflammation and septic shock in animals.

Three kinds of LPS modifications have been observed: (1) substitution of the phosphate groups in lipid A with phosphoethanolamine, (2) decoration of the basic structure with additional sugar residues, and (3) addition of palmitate by esterification. Derivatization with phosphoethanolamine renders the bacteria resistant to a lipid A-binding, cyclic, cationic peptide antibiotic, polymyxin, while palmitoylation provides resistance against cationic antimicrobial peptides induced by the innate immune system in response to bacterial infections. These modifications may occur alone or in combination on a single LPS molecule, yielding multiple LPS species in a single bacterium.

Gram-negative bacteria produce OM blebs or vesicles of 0.5–1.0 μm in diameter. These vesicles that can contain enzymes and signaling molecules are released into the culture medium to be delivered to other bacteria, where the vesicles again fuse to the OM of the recipient bacterium. These vesicles can also be used to deliver bacterial protein toxins to mammalian cells. They provide a novel mechanism of prokaryotic communication.

OM Proteins

OMPs are usually synthesized in the bacterial cytoplasm as precursors with N-terminal signal peptides and are then translocated across the CM via the general Sec pathway. After removal of the signal peptides by a signal peptidase, many of the mature proteins insert themselves into the OM and assume β-barrel structures with hydrophobic, membrane-embedded outer surfaces suitable for interaction with LPS and membrane lipids. OM proteins can be classified based on their functions: (1) lipoproteins, (2) general porins, (3) substrate-specific receptors, (4) enzymes, and (5) various other OM proteins.

Lipoproteins

Dozens of lipoproteins have been described. The murein lipoprotein, Lpp of *E. coli*, is the most prominent and best-studied member. Lpp is a small protein (7200 Da) present in about a million copies per cell. Its N-terminal cysteine is modified at two sites. The cysteyl sulfhydryl group is substituted with a diglyceride, and the α-amino group is derivatized by a fatty acyl residue. This allows penetration into and anchoring to the inner leaflet of the OM. About one-third of these molecules are bound covalently to the underlying peptidoglycan cell wall layer, thereby attaching the OM to the wall. Deletion of the *lpp* gene results in numerous defects such as leakage from the periplasm, increased susceptibility of the cell to toxic compounds, and increased blebbing of membrane vesicles from the OM with the release of vesicles into the external milieu. These lipoproteins thus serve important structural roles.

Porins

Porins allow the diffusion of fairly small hydrophilic (and occasionally hydrophobic) molecules. They exhibit varying degrees of substrate specificity. They can be nonspecific or show selectivity only for the charge of the substrate, either anionic or cationic. Some are even specific for certain types of molecules – oligosaccharides, peptides, amino acids – or anions. They generally form OM water-filled channels. Many are either monomeric or homotrimeric, the latter being formed by three hollow β-barrels. However, other quaternary porin structures have been reported. They can be small or large, having

8–24 transmembrane β-strands per polypeptide chain, and they frequently have extra hydrophilic protein domains on one or both sides of the membrane. Their pore sizes vary; several of their three-dimensional structures have been determined, allowing visualization of the permeation pathway. A conspicuous structural feature is the presence of an 'eyelet' region, a narrow constriction in the pore, lined with charged residues. These charged residues determine in part the specificity of the porin for the substrates.

As an example, the trimeric phosphoporin, PhoE of *E. coli*, is produced under conditions of phosphate starvation. The channel-forming motif of PhoE is a 16-strand antiparallel β-barrel. Short β-hairpin turns define the periplasmic side of the barrel, whereas long irregular loops are found at the cell surface. PhoE functions primarily in anion transport due to the presence of positively charged residues near the mouth of the channel. OMPs probably fold in the periplasm before being inserted into the OM in the presence of LPS. The insertion of proteins into these membranes is generally poorly understood, but it depends on a multicomponent protein insertion apparatus which is essential for the process (see 'OM protein insertion').

Substrate-specific receptors

While most nutrients gain access to the periplasm by diffusion through porins, a few substrates are too large to enter by this route. Large receptor/transport systems form energy-dependent gated channels which take up these compounds. The TonB/ExbB/ExbD type systems energize transport using the PMF- and TonB-dependent receptors. Examples of such receptors in *E. coli* include BtuB for vitamin B_{12} uptake and several receptors for the uptake of different iron–siderophore complexes. Iron–siderophore complexes are high-affinity iron chelators of microbial origin. Transport requires an interaction with the periplasmic protein TonB, which may shuttle between the inner and outer membranes. The action of TonB requires an energized CM in the form of a PMF. Energy is transferred to the receptors with the assistance of the two cytoplasmic H^+ channel-forming membrane proteins ExbB and ExbD, which energize TonB by transporting protons down their electrochemical gradient, across the CM. Energized TonB then transmits its energy to the receptors.

These siderophore receptors and BtuB are β-barrel monomeric proteins that consist of 22 transmembrane β-strands each. The N-terminal domain consists of a globular structure that inserts itself into the barrel from the periplasmic side, forming a plug. Binding of a ligand induces a conformational change in the protein so that the most N-terminal portion containing a short motif, called the 'TonB box', can interact with the TonB protein. This first step is followed by a large-scale conformational change caused by the energized TonB. The molecular details of this process are not yet fully understood.

OMP Insertion

Gram-negative bacterial OMPs are assembled from the periplasm into the OM in a process that has only recently become a subject of molecular research. Large (~800 aas) OMPs, complexed with several others, play a crucial role. These bacterial proteins are very distantly related to the chloroplast import-associated channel proteins, IAP75, constituents of the chloroplast envelope protein translocase. IAP75 has been shown to be a β-barrel porin in the OM of plant chloroplasts. Another homologue is the yeast mitochondrial sorting and assembly machinery (SAM) constituent, SAM50. The SAM complex in yeast mitochondria consists of at least three proteins and is required for the assembly of OM β-barrel proteins in mitochondria. It seems clear that these organellar protein complexes were derived from bacterial proteins when endosymbiotic α-proteobacteria and cyanobacteria became permanent residents of eukaryotic cells as mitochondria and chloroplasts, respectively.

The functionally characterized homologue in the Gram-negative bacterium *Neisseria meningitidis* is essential for bacterial viability. It has a two-domain structure with an N-terminal periplasmic domain rich in hydrophilic repeat sequences and a C-terminal domain that forms an integral OM β-barrel. Unassembled forms of various OMPs accumulate when Omp85 is depleted. Homologues of Omp85 are present in all Gram-negative bacteria examined, but not in other prokaryotes. The *E. coli* homologue functions as a principal constituent of a complex that catalyzes protein insertion into the OM.

Normally OMPs are translocated into the periplasm via the Sec translocase. They are believed to fold in the periplasm before being inserted into the OM. Folding is stimulated by small periplasmic chaperone proteins. In *E. coli*, these chaperones feed a substrate protein to the OM integrated multiprotein complex required for OM biogenesis. It is probable that the activities of this complex are absolutely required for OMP assembly. The specific biochemical roles of the individual protein constituents have not yet been determined.

OMs of Acid-Fast Gram-Positive Bacteria

Acid-fast bacteria belong to a distinctive suprageneric actinomycete taxon, which includes mycobacteria, corynebacteria, nocardia, rhodococci, and other closely related genera. All of these bacteria share the property of having an unusual cell envelope composition and architecture (**Figure 2**(**a**)). Based on available published data, the envelope layers consist of a typical CM of phospholipid and protein, a characteristic wall of unusual structure, and a complex outer layer. Although studies with mycobacteria are more detailed than with other related genera, it is evident that the envelopes of these related bacteria are all similar, especially in terms of ultrastructure and cell-wall composition.

The cell walls of these bacteria are formed by thick *meso*-diaminopimelic acid-containing peptidoglycan layers covalently linked to arabinogalactan. The arabinogalactan is in turn esterified with long-chain α-alkyl,

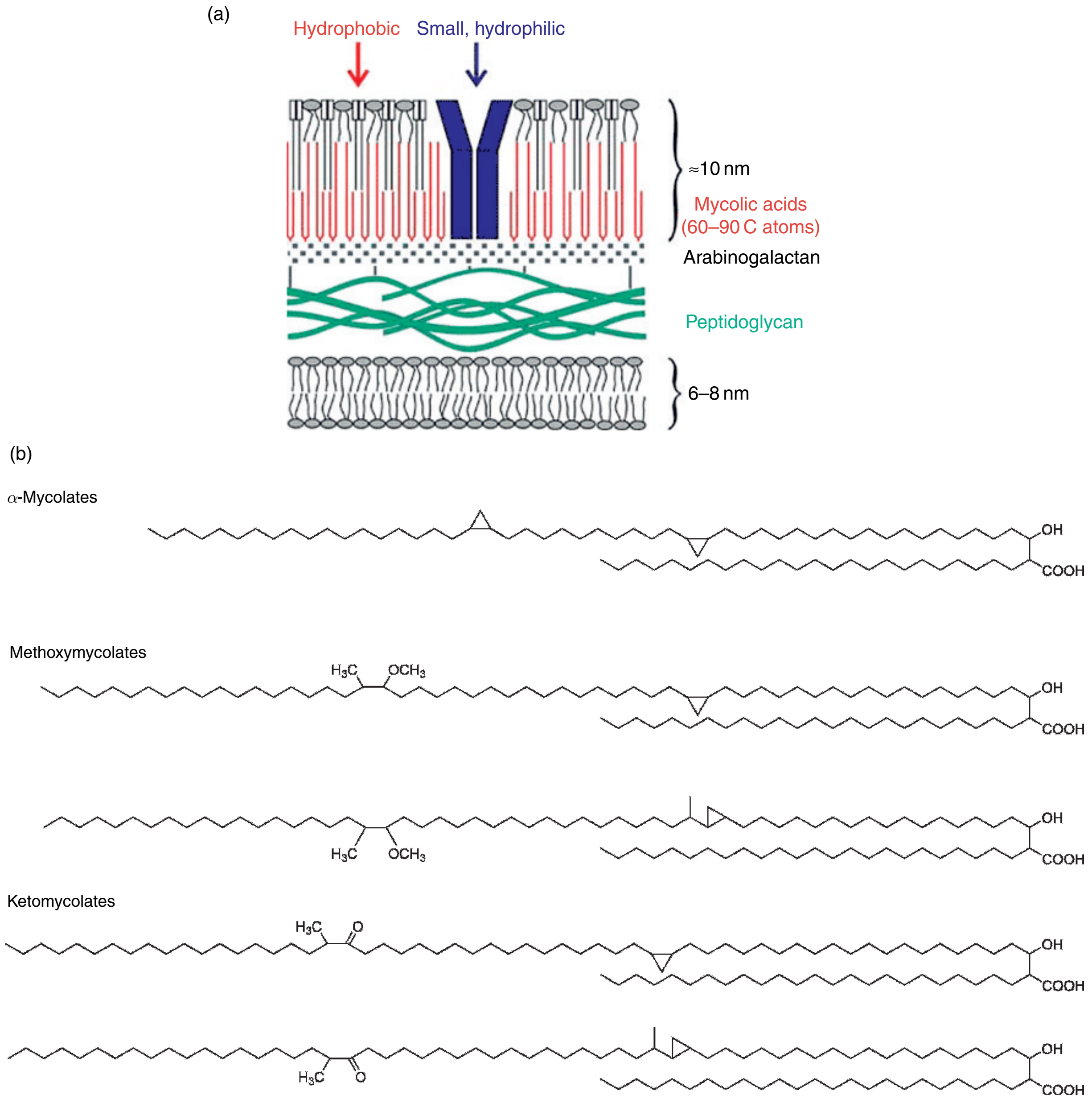
(a)
Hydrophobic
Small, hydrophilic
≈10 nm
Mycolic acids
(60–90 C atoms)
Arabinogalactan
Peptidoglycan
6–8 nm
(b)
α-Mycolates
OH
COOH
Methoxymycolates
H_3C
OCH_3
OH
COOH
H_3C
OCH_3
OH
COOH
Ketomycolates
H_3C
O
OH
COOH
H_3C
O
OH
COOH

Figure 2 (a) The most important structural components of the mycobacterial cell envelope. While small hydrophobic molecules may diffuse through the outer lipid bilayer, small hydrophilic molecules require the involvement of outer membrane porins as indicated at the top of the diagram. The figure illustrates the covalent linkages between cell wall peptidoglycan, arabinogalactan, and mycolic acids. (b) The structures of mycolic acids in *Mycobacterium tuberculosis*. α-Mycolates: the meromycolate chains contain two *cis*-cyclopropanes; methoxymycolates: their meromycolate chains contain an α-methyl-ether moiety in the distal position and a *cis*-cyclopropane or an α-methyl *trans*-cyclopropane in the proximal position; ketomycolates: their meromycolate chains contain an α-methyl ketone moiety in the distal position and proximal functionalities as in the methoxy series. Unsaturations are present in some meromycolate chains of *M. tuberculosis* (not shown).

β-hydroxy fatty acids. These fatty acids in mycobacteria are called mycolic acids (**Figure 2(b)**). They possess very long chains (C_{60-90}) and may contain various branches, oxygen functions such as hydroxyl, methylated hydroxyl, and keto groups as well as unsaturations. Mycolic acids found in other actinomycetes consist of mixtures of saturated and unsaturated acids, but they contain shorter chains. Nocardomycolic acids are of length C_{40-50} while corynomycolic acids are of C_{22-36}. Thus, mycobacterial OMs are thicker than nocardial OMs which, in turn, are thicker than corynebacterial OMs.

Acid-fast high G+C Gram-positive microbes share with Gram-negative bacteria the property of possessing OMs that are very different in composition from the plasma membranes. While the outer barrier in Gram-negative bacteria is a typical bilayer of phospholipid and LPS, in mycobacteria, nocardia, and corynebacteria, the cell wall-linked mycolates comprise much of this barrier. The lengths and structures of mycolic acids are important in determining not only the membrane width, but also the envelope fluidity and permeability. The existence of OM diffusion barriers in mycobacteria, corynebacteria, and nocardia is reinforced by the characterization of cell envelope proteins with pore-forming abilities. The OMs of some of these organisms are essential for many of their pathogenic properties.

In all currently proposed models, the outer permeability barrier of mycobacteria consists primarily of a monolayer of mycoloyl residues covalently linked to cell wall arabinogalactans (**Figure 2(a)**). Other lipids may be arranged in an outer leaflet to form a complex asymmetric bilayer. The structural details of this bilayer are yet to be fully elucidated.

Freeze-fractured samples of mycobacteria, corynebacteria, and other related bacteria have revealed details of the envelope structures of these organisms with distinct lipid domains. Freeze-fracture electron microscopy also revealed the presence of ordered arrays on the surfaces of these envelopes consisting of surface layer proteins (S-layers) that overlie the OMs. There may therefore be five layers: (1) the inner CM, (2) the cell wall, (3) the arabinogalactan/arabinomannan polysaccharide layer, (4) the OM, and (5) the external proteinaceous S-layer. All have protective functions.

The five layers of the acid-fast bacterial envelope are believed to be integrated to form the protective envelope as follows. Immediately outside of the CM, the cell wall peptidoglycan layer is covalently linked to the arabinogalactans, and these are esterified with mycolic acids. Because the amounts of cell wall-linked mycolates are insufficient to cover the entire bacterial surface, other types of noncovalently bound lipids must play roles in forming the OM. In fact, these lipids have been shown to form bilayer structures spontaneously. Thus, the cell wall permeability barriers in these bacteria involve both covalently wall-linked mycolates and noncovalently bound lipids. These molecules together with various proteins comprise the bulk of the cell envelopes.

OMs of Mycobacteria: Function, Structure, and Composition

As noted above, the permeability of mycobacteria, and other bacteria related to them, to substances in their environments is determined by the properties of their envelopes. Current models depicting the structural organization of the mycobacterial cell wall assume that peptidoglycan and arabinogalactan strands overlie the CM forming horizontal layers beneath perpendicularly oriented mycolic acids. The mycolate layer prevents entry of small hydrophilic molecules which gain access to the cell only via porins (**Figure 2(a)**). Some small lipophilic molecules may diffuse through the lipid layer. The capsule prevents passage of virtually all macromolecules unless specific transport systems mediate their entry or exit. The structure of the outer lipid barriers is similar in all mycobacteria, but the capsule is more abundant in slow-growing species than in fast-growing species. The slow-growing organisms comprise the group that includes most mycobacterial pathogens.

Mycobacteria secrete proteins that are important to the pathogenesis of the many human and animal diseases caused by these microbes. Information about how the secreted proteins and the polysaccharides of the capsule cross the outer lipid barrier is fragmentary and is only now coming to light. It is possible that proper knowledge of mycobacterial envelope permeability will enable new approaches to the treatment of mycobacterial diseases.

The cell envelopes of mycobacteria substantially contribute to their resistance to therapeutic agents. This is largely due to the presence of the C_{60-90} mycolic acids that are covalently linked to the large arabinogalactans as well as the acylated and nonacylated arabinomannans. Recent studies have clarified the unusual structures of arabinogalactans as well as extractable cell wall lipids such as phenolic glycolipids, glycopeptidolipids, and trehalose-based lipooligosaccharides called ‘cord factor’. Most of the hydrocarbon chains of these lipids assemble to produce the exceptionally thick, asymmetric OM. Structural considerations suggest that the fluidity is unusually low in the innermost parts of bilayer, gradually increasing toward the outer surfaces. Differences in mycolic acid structure may affect the fluidity and permeability of the bilayer and explain the different sensitivities of various mycobacterial species to lipophilic compounds. Hydrophilic nutrients, vitamins, minerals, toxins, and growth inhibitors, in contrast, traverse the OM exclusively via porin channels.

The detailed molecular structures of mycobacterial cell envelopes and their lipids are currently coming to light (see **Figures 2(a)** and **2(b)**). The cell wall architecture resembles a massive 'core' comprised of peptidoglycan covalently attached via a linker unit (L-Rha-D-GlcNAc-P) to a linear galactofuran. This in turn is attached to several strands of a highly branched arabinofuran, which is attached to mycolic acids. The mycolic acids are perpendicularly oriented relative to the plane of the membrane (**Figure 2(a)**). They create a lipid barrier responsible for many of the physiological, disease-inducing, and drug resistance properties of *Mycobacterium tuberculosis, Mycobacterium leprae, Mycobacterium bovis, Mycobacterium avium*, and other mycobacterial pathogens.

Intercalated within this envelope are the lipids that have intrigued biochemists for over five decades: the phthiocerol dimycocerosate, cord factor (dimycolyltrehalose), the sulfolipids, the phosphatidylinositol mannosides (PIMs) and others. The lipomannans and lipoarabinomannans also play important roles in the physiology and pathogenesis of mycobacteria. These molecules have functions of signaling to the host and stimulating immune responses of infected humans and animals. Mycolic acids are recognized by CD1-restricted T-cells, and antigen 85 – one of the most powerful protective antigens of *M. tuberculosis* – is a mycolyltransferase. Moreover, lipoarabinomannans, when 'capped' with short mannose oligosaccharides, promote phagocytosis of the bacteria by animal cells, an important phase of pathogenesis.

Sequencing of the *M. leprae, M. tuberculosis* and other pathogenic and nonpathogenic mycobacterial genomes has aided efforts to define the biosynthetic pathways for all of these exotic lipid and complex carbohydrate-containing molecules. These include mycolic acids, the mycocerosates, phthiocerol, lipidated arabinomannans and arabinogalactans, and the polyprenyl phosphates. We now know that synthesis of the entire core is initiated on a decaprenyl-P with synthesis of linker units. There seems to be concomitant extension of the galactan and arabinan chains while these intermediates are transported through the CM. The final steps in these events, the attachment of mycolic acids, and ligation to peptidoglycan, must occur in the periplasm. Elucidation of these complicated processes awaits definition.

Mycolic Acids and Other Unusual Mycobacterial Lipids

As noted above, mycolic acids of mycobacteria are long-chain fatty acids, many of which vary in size and structure (**Figure 2(b)**). Arabinogalactan mycolates are covalently linked via phosphodiester linkages to the underlying peptidoglycan cell wall polymer (**Figure 2(a)**). Mycolic acids together with other cell wall lipids (trehalose-based lipooligosaccharides, phenolic glycolipids, and glycopeptidolipids) comprise part of the OM and contribute to the low permeability of these envelopes. They account in part for the remarkable drug resistance of mycobacteria rendering treatment of mycobacterial diseases difficult. This is particularly important to human health since one-third of the world's population is infected with mycobacteria, and millions die from mycobacterial diseases every year. The hydrophobic hydrocarbon chains of these lipids comprise the OMs which may be the thickest of all biological membranes yet identified. The asymmetric OM consists largely of long-chain mycolic acids comprising most of the inner leaflet with a diversity of other lipids contributing to the outer leaflet.

Some lipids and lapidated glycans unique to mycobacteria appear to be present in both the inner and the outer membranes. These are the PIMs, their hypermannosylated derivatives, lipomannans, and lipoarabinomannans. They are important virulence factors in pathogenic species. These facts reveal a surprising degree of lipid diversity in mycobacterial envelopes and show that fatty acyl esters linked to complex carbohydrates contribute to the rigidity of these structures.

Mycobacterial OMPs

OMPs of mycobacterial species are far less well characterized than those of Gram-negative bacteria; however, substantial progress has been made. The most important observations are summarized here.

Lipoproteins

The availability of complete genome sequences of mycobacterial species has greatly facilitated the identification of OM lipoproteins. The occurrence of genes encoding these lipidated proteins in the reduced-size genome of *M. leprae* provides a guide to the minimal mycobacterial gene set. Surprisingly, perhaps, these lipoproteins are superficially similar to those of Gram-negative bacteria.

The consensus sequence at the N-terminal region of these proteins includes the cysteine residues to which the lipid moiety becomes attached. This sequence provides clues for the identification of these proteins. More than 20 potential lipoprotein genes have been identified in the *M. leprae* genomic sequence. Lipoprotein LpK, for example, encodes a 371 amino acyl precursor protein which becomes lipidated after it is synthesized, exported from the cytoplasm and proteolytically processed. The purified lipoprotein induces production of interleukin-12 (IL-12) in humans. This implies that LpK is involved in protective immunity against leprosy. The pursuit of such lipoproteins is likely to reveal details of the pathogenesis of a variety of mycobacterial diseases.

Mycobacterial OM porins

Pore proteins in Gram-negative bacterial OMs that span the membrane mediate the diffusion of small hydrophilic nutrients such as sugars, amino acids, anions, cations, and vitamins (see 'Porins'). *M. tuberculosis* possesses at least three porins, one which is the low-activity channel protein, OmpATb. OmpATb is essential for adaptation of *M. tuberculosis* to low pH and survival in macrophage. The channel activity of OmpATb probably plays a role in the defense of *M. tuberculosis* against acidification within the phagosomes of macrophage. In contrast to other acid-fast bacterial porins, it shows sequence similarity to OMP (OmpA) homologues from Gram-negative bacteria.

Another porin, MspA, is the main porin of the related, fast-growing, nonpathogenic species *M. smegmatis*. It forms a tetrameric complex with a single central pore of 10 nm length and has a cone-like structure. This structure is entirely different from that of the trimeric porins of Gram-negative bacteria suggesting that these two porin types evolved independently. In agreement with this conclusion, MspA shows no significant sequence similarity with any of the Gram-negative bacterial OMPs. The approximately 50-fold decreased numbers of porins in acid-fast bacteria compared with Gram-negative bacteria and the increased lengths of mycobacterial pores are two primary determinants of the low permeabilities of outer mycobacterial membranes to small hydrophilic solutes. Slow transport through porins needs to be considered when designing novel drugs against mycobacterial diseases.

Compartmentalization of Lipid Biosynthesis in Mycobacteria

The plasma membranes of *Mycobacterium* species are the sites of synthesis of several distinct classes of lipids. Some of these lipids are retained in the inner membrane while others are exported to the overlying cell envelope. Enzymes involved in the biosynthesis of different major lipid classes, the PIMs and aminophospholipids, for example, are compartmentalized within the CM and the cell wall containing envelope fractions. Enzymes involved in the synthesis of early PIM intermediates are localized to a plasma membrane subdomain, while later stages of synthesis seem to be associated with external parts of the envelope. This suggests that like the LPSs in Gram-negative bacteria, complex outer envelope lipids may be synthesized in several stages that occur in different compartments of the cell envelope.

Molecular Action of Antimycobacterial Agents

There is evidence that many drugs exert their antimycobacterial activities by interacting with classical bacterial proteinaceous targets. This possibility is supported by both direct and indirect evidence. Mechanistic studies have been performed for drugs such as fluoroquinolones, macrolides, rifampicin, and streptomycin. Although the modes of action of many agents with antimycobacterial activities are not well understood, it seems likely that many drugs will prove to inhibit specific molecular targets involved in the biosynthesis of mycobacterial cell envelope constituents such as its many unique lipids and carbohydrate polymers. The recent reemergence of tuberculosis as an important human pathogen has prompted the development of improved methods for exploring the structures, biochemistry, and genetics of mycobacterial envelopes. These advances should be useful in gaining a better understanding of the molecular basis of drug action in mycobacteria.

Archaeal Membranes

Archaea are similar to bacteria in many aspects of cell structure, but they differ radically with respect to the lipid compositions of their membranes (**Figures 3(a)** and **3(b)**). The structures of their cell surfaces and their protein, lipid, and carbohydrate constituents are also unique. Archaeal membranes contain glycerol ether-linked lipids rather than ester-linked lipids as are found in bacteria and eukaryotes (**Figure 3(b)**). These lipids are based on isoprenoid side chains. In addition, bacterial-type peptidoglycan cell walls are altogether lacking, and in their place, cell walls consisting of surface layer proteins are present (**Figure 3(a)**). OMs are not found in the better-characterized archaea, although they have been identified in one class of these organisms and are probably present in others. Because the existence and the unique properties of these organisms have been recognized for a relatively short period of time, much less is known about the OMs of archaea than about those of Gram-negative and acid-fast Gram-positive bacteria as well as those of eukaryotic organelles.

Archaeal Lipids

Polar ether lipids of archaea account for 80–90% of the total membrane lipids in these organisms. The remainder are neutral squalenes and other isoprenoids. Many such unique lipids have been discovered in recent years. Genus-specific combinations of various lipid core structures include diether-tetraether, dietherhydroxydiether, and diether-macrocyclic diether-tetraether lipid moieties. The basic structure of a representative archaeal ether lipid and its comparison with a bacterial eukaryotic ester lipid are shown in **Figure 3(b)**.

Some archaeal species have only the standard diether core lipids. None are known with predominantly tetraether lipids present in certain sulfur-dependent

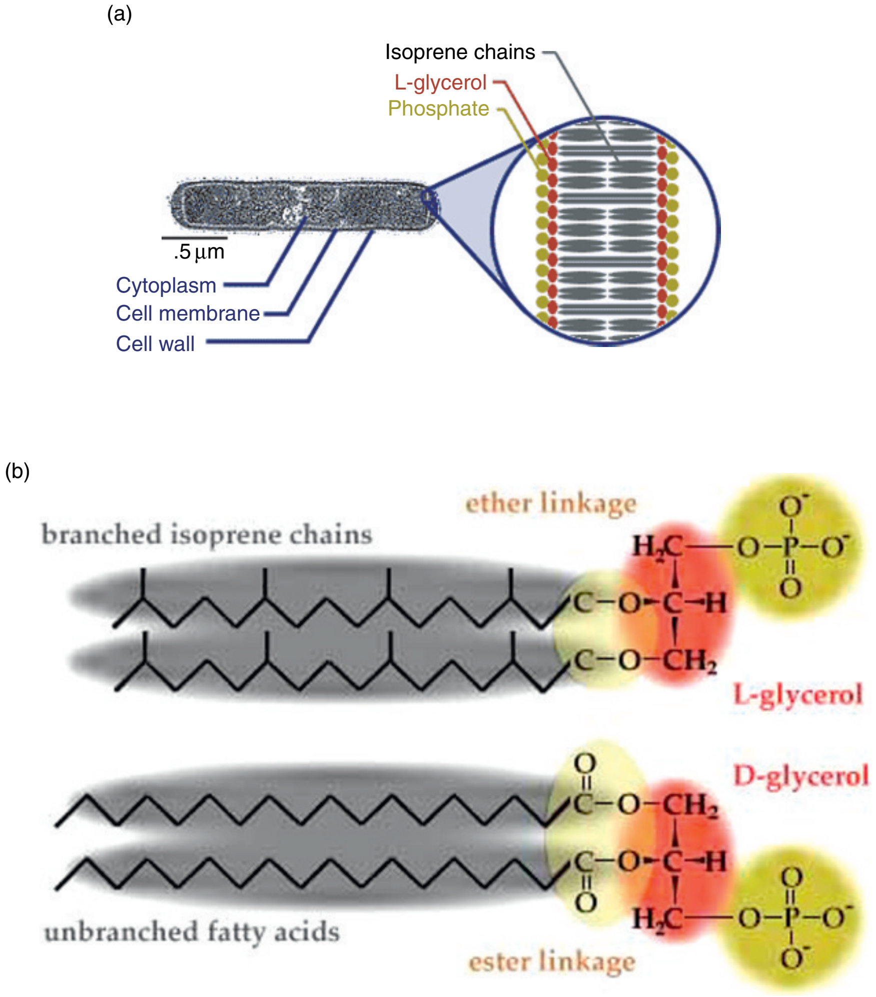

Figure 3 (a) A typical archaeal cell illustrating the positions and structural features of the ether-linked lipids in the cytoplasmic membrane. The illustrated membrane is a 'blow-up' of the archaeal cell as visualized by electron microscopy. (b) Structure of an archaeal ether lipid (top) compared with that of a typical bacterial ester lipid (bottom). Primary structural differences are illustrated.

archaea. The relative proportions of these lipid cores are known to vary with growth conditions in some archaea such as *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*. Polar headgroups in glycosidic or phosphodiester linkage to glycerol consist of polyols, other carbohydrates, and amino compounds. The available structural data indicate close similarities between the polar lipids found in species of the same genus. Thus, the closer the phylogenetic relationships of the organisms, the more similar the lipid compositions of their membranes. These ether-containing lipid structures are more stable than the ester-containing lipids of bacteria and eukaryotes. This may have resulted in part through evolution, from the extreme environments inhabited by many archaea.

The extreme environments that some archaea thrive in include hot springs and strongly acidic, salty, and/or alkaline lakes. For example, *M. jannaschii* grows optimally at 85 °C and pH 6, *Thermoplasma acidophilum* at 55 °C and pH 2, and *Halobacterium salinarum* in near-saturated salt brines. Archaea that can not only survive but also grow at temperatures above 100 °C are known.

A primary role of any cell CM is to provide a selective barrier between the external environment and the cytoplasm. Given the extreme environmental conditions conducive to archaeal growth, it is not surprising that their membranes contain lipids that differ markedly from those of bacteria and eukaryotes. The presence of ether rather than ester bonds contributes to their chemical stability, particularly at high temperatures and extreme pH values. Surprisingly, the glycerol ethers of archaea contain an *sn*-2,3 stereochemistry that is different from that of the *sn*-1,2 stereochemistry of glycerophospholipids of the other domains of life. The unique basic lipid core structures of these two lipid types are depicted in **Figure 3**(**b**).

Two major classes of archaeal lipids include the archaeol lipids (diphytanyl glycerol diethers) and the caldarchaeol lipids (dibiphytanyl diglycerol tetraethers). The caldarchaeol lipids span the membrane, and liposomes made from these lipids preferentially form monolayers rather than the bilayers formed from conventional glycerophospholipids. Many of the tetraether lipids are phosphoglycolipids containing one or more sugar residues at one pole, most commonly gulose, glucose, mannose, and/or galactose, and a phosphopolyol moiety, such as phosphoglycerol or inositol, on the other. The more bulky sugar residues probably face outward, and the phosphate residue may face toward the cytoplasmic side of the membrane. Depending on the growth temperature, certain thermophilic archaea are capable of controlling membrane fluidity by altering the number of cyclopentane rings (from 0 to 8 in caldarchaeol lipid chains).

Archaeal OMs

Many hyperthermophilic *Crenarchaeota* have two-dimensional crystalline arrays of (glyco-)protein subunits (the S-layer) as the more rigid component of their cell walls. In most cases, these protein arrays constitute the outermost surfaces of the cells. The subunits themselves are directly anchored to the CM by stalk-like structures. The space between the CM and the S-layer is called the 'quasi-periplasmic space' by analogy to the equivalent structures in bacterial cell envelopes.

Ignicoccus is a hyperthermophilic archaeon belonging to the *Desulfurococcales* subdivision of the *Crenarchaeota*. It is a chemolithoautotrophic organism that obtains its energy by the reduction of elemental sulfur with molecular hydrogen. Cells of *Ignicoccus* have been examined ultrastructurally following cultivation in cellulose capillaries and processing by high-pressure freezing. They consistently showed a cell envelope structure previously unknown among the archaea: CM and OM separated by a periplasmic space of variable width (20–200 nm) containing membrane-bound vesicles. The outer sheath, approximately 10 nm wide, seemed to resemble the OMs of Gram-negative bacteria. The *Ignicoccus* sheath contains three types of particles: (1) numerous irregularly packed single particles, about 8 nm in diameter; (2) putative pores with a diameter of 24 nm; and (3) tiny particles arranged in a ring with a diameter of 130 nm surrounding the pores. Clusters of up to eight particles, each particle 12 nm in diameter, were conspicuous. Freeze-etched cells exhibited a smooth surface without a regular pattern, with frequent fracture planes through the outer sheath. This observation indicated to the researchers the presence of an OM and the absence of an S-layer. The study illustrated the novel complex architecture of the cell envelope of *Ignicoccus*. Comparative studies suggest that OMs of prokaryotes have evolved independently at least three times, once in Gram-negative bacteria, once in high G+C Gram-positive bacteria, and once in *Crenarchaeota*.

Membrane Transfer Between Cells

Ignicoccus lives in symbiosis with another archaeon, a very small, single-celled organism called *Nanoarchaeum equitans* (see **Figure 4**). The *Nanoarchaeum* cell has one of the smallest genomes yet sequenced (less than 500 000 bp). In fact, too few genes are present to code for all of the biological functions thought to be essential for life. It can live only together with *Ignicoccus*. Among the missing functions are the enzymes that catalyze lipid biosynthesis. If these enzymes are really absent from this organism, then how does *Nanoarchaeum* get its lipids for construction of its CM?

Ultrastructural analyses reveal not only the two-membrane envelope of *Ignicoccus*, but also the presence of intraperiplasmic vesicles. Because the lipid and protein compositions of the inner and outer membranes are different, it has been possible to establish that these vesicles derive from the inner membrane of *Ignicoccus*. Moreover, the lipids in the nanoarchaeal membrane are very similar, if not identical, to those in the CM of *Ignicoccus*.

These observations led to the postulate that one organism makes the lipids for both. Some of the membrane

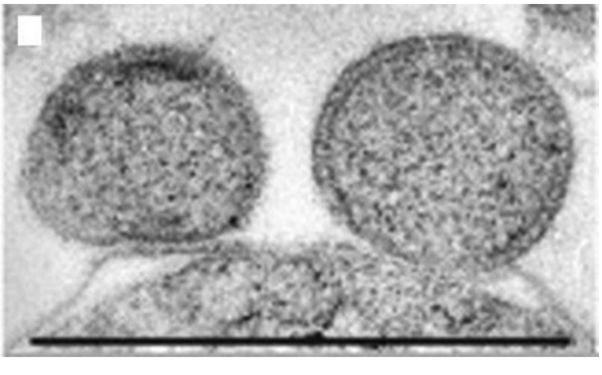

Figure 4 An electron microscopic depiction of an *Ignicoccus* cell (bottom), showing the inner and outer membranes, in symbiotic association with two *Nanoarchaeum* cells (top).

transport proteins in the nanoarchaeal membrane of one organism may also derive from its symbiotic partner cell. The details of the transfer process, which still need to be confirmed, are yet to be established. However, it already seems likely that these symbiotic archaea have developed mechanisms for intercellular communication and molecular transfer involving periplasmic vesicles that are unique to them. Alternatively, elucidation of such mechanisms may lead to the discovery of analogous processes in bacteria and eukaryotes.

Conclusions

Prokaryotes, including bacteria and the much less well-studied archaea, possess cell envelopes of extremely varied compositions and structures. In both prokaryotic domains of living organisms, as in organelles of eukaryotes, the envelopes can possess one or two membranes. The two membranes always consist of different combinations of lipids and proteins. We are now coming to appreciate the complexities of the assembly machineries that function to construct these envelopes. Many are present in specific membranes while others span the entire cell envelope structures. Moreover, completely different transport apparati are found in the inner and outer membranes of organisms that have both. Evaluation of the structural data available for the OMs of Gram-negative bacteria, high G+C Gram-positive bacteria, and archaea leads to the conclusion that prokaryotic OMs have probably evolved independently in these three organismal types. It is clear that further research will be required to clarify the many important but poorly understood issues dealing with basic aspects of the functions, structures, biogenesis, and evolution of prokaryotic OMs. Moreover, novel processes and mechanisms are likely to come to light. This chapter thus serves as a progress report of prokaryotic membrane research efforts that will hopefully provide a basis for future advances.

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Cell Structure, Organization, Bacteria and Archaea

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Defining Statement
Characteristics of Prokaryotes
Cell Envelope – Appendages
Cell Envelope
Cytoplasm
The Nucleoid
Further Reading

Glossary

cell envelope The boundary that envelopes a cell. It is composed of a cytoplasmic membrane and a cell wall.

cryo-electron tomography Frozen biological objects are imaged in an electron microscope under various viewing angles. Image processing results in a 3D representation. This, in turn, can be used to obtain virtual sections through the object.

flagellum A proteinaceous filament of several micrometer length that enables bacterial motility. Through a basal body, it is integrated into the cell envelope. Proton motive force drives a rotor located in a stator.

hamus Plural: hami. Proteinaceaous filaments on the surface of an archaeal organism SM1. They terminate into fishhook-like structures.

nucleoid The bacterial or archaeal chromosome.

nucleoplasm The DNA-containing region in the bacterial or archaeal cell.

penicillin-binding proteins Proteins involved in bacterial peptidoglycan assembly outside the cytoplasmic membrane. They bind specific antibiotics.

peptidoglycan A bacterial wall component built from glycan chains interconnected by cross-linked peptide side chains. The glycan chains are made up of disaccharide units, which are composed of *N*-acetylglucosamine and *N*-acetylmuramicacid. Cross-linking through the peptide side chains provides for a strong network.

pili Proteinaceous filaments on a bacterial cell surface that can adhere to other organisms, notably eukaryotic epithelia. Their dimensions are markedly smaller than those of flagella.

pseudopeptidoglycan Also denoted as pseudomurein. The peptidoglycan equivalent of some archaeal species. It contains no D-amino acids. The disaccharide units of the glycan chains are composed of *N*-acetylglucosamine and *N*-acetyltalosaminuronic acid.

sacculus A covalently closed structure that has the shape of a bacterium. It is composed of glycan chains that are interconnected by peptide side chains.

S-layer A protein layer of regularly arranged subunits on the surface of a prokaryote.

Abbreviations

CM	cytoplasmic membrane
CW	cell wall
CY	cytoplasm
ENV	envelope surrounding the nucleoid
F	flagellum
GFP	green fluorescent protein
GlcNAc	*N*-acetylglucosamine
ICM	intracytoplasmic membrane
IWZ	inner wall zone
L	L-ring in outer membrane
LPS	lipopolysaccharide
MS	MS-ring in cytoplasmic membrane
MurNAc	*N*-acetylmuramic acid
N	nucleoid
NacTalNA	*N*-acetyltalosaminuronic acid
NU	nucleoid
OM	outer membrane
OWZ	outer wall zone
P	pilus
P	P-ring in peptidoglycan layer
PA	paryphoplasm
PE	periplasm
PF	proteinaceous filaments
PG	peptidoglycan
PR	polyribosome
TA	teichoic acid

Defining Statement

The structure of bacteria and archaea is presented here with some extra focus on the latter. The rationale has been to discuss the cell structure, while going from the outside of the cell to the inside, that is, from cellular appendages to the nucleoid. Special emphasis has been placed on novel results obtained by cryo-electron tomography.

Characteristics of Prokaryotes

An Overview of Prokaryotic Structure

Prokaryotes are divided into the domains Bacteria (formerly Eubacteria) and Archaea (formerly Archaebacteria). As prokaryotes (*pro*, before and *karyon*, nucleus), they contain no membrane-enveloped genome. This common feature implies direct contact between DNA and cytoplasm. In contrast to the situation in eukaryotes, transcription and translation occur in one and the same compartment. Thus, while genes are being transcribed, ribosomes position themselves on the emerging messenger RNA and protein synthesis takes place simultaneously. In case of synthesis of a membrane protein, a continuous, be it transient, connection exists between DNA and membrane (**Figure 1**).

So far, prokaryotic structure has been mainly studied in bacteria. Though many species have been investigated, some organisms have been used as models for biochemical, genetic, physiological, and ultrastructural research. *Escherichia coli, Salmonella typhimurium*, and *Caulobacter crescentus* have dominated the Gram-negative field, whereas *Bacillus subtilis, Enterococcus* sp., *Pneumococcus* sp., *Staphylococcus aureus*, and *Streptococcus* spp. are popular Gram-positives. The distinction between Gram-positive and Gram-negative organisms makes sense, because in the case of bacteria, it reflects a basic difference in cell wall architecture (**Figure 2** and see below). Model organisms have advantages and disadvantages. Clearly, concerted efforts of many groups augment progress in specific scientific fields. Nevertheless, interesting biological phenomena in other organisms may not be noticed for a long time. For instance, it has been found quite recently that an organism like *Vibrio cholerae* has specific DNA segregation mechanisms for its two different chromosomes.

Recent research has demonstrated a remarkable membrane compartmentalization in some planctomycete species. In these bacteria, which lack peptidoglycan, the nucleoid region is enveloped by a single or a double membrane. In *Gemmata obscuriglobus*, a double membrane shields a DNA- and ribosome-containing area from a cytoplasmic area also containing ribosomes (**Figure 3**). This area is bounded by an intracytoplasmic membrane. Between the intracytoplasmic membrane and the

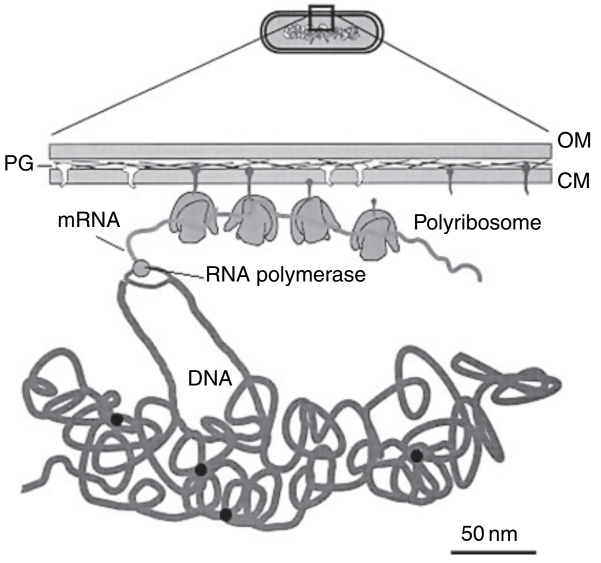

Figure 1 Structural continuity between the nucleoid and the envelope through cotranscriptional biosynthesis of membrane proteins. CM, cytoplasmic membrane; OM, outer membrane; PG, peptidoglycan. Reproduced from Woldringh CL, Jensen PR, and Westerhoff HV (1995) Structure and partitioning of bacterial DNA: Determined by a balance of compaction and expansion forces? *FEMS Microbiology Letters* 131: 235–242.

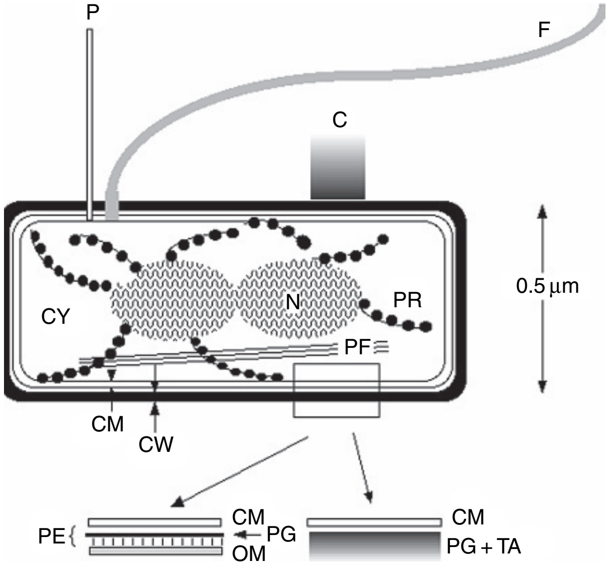

Figure 2 Schematic representation of a bacterium. The Gram-positive (right) and Gram-negative (left) cell envelopes are indicated below. C, capsule; CM, cytoplasmic membrane; CW, cell wall, CY, cytoplasm; F, flagellum; N, nucleoid; OM, outer membrane; P, pilus; PE, periplasm; PF, proteinaceous filaments; PG, peptidoglycan; PR, polyribosome; TA, teichoic acid.

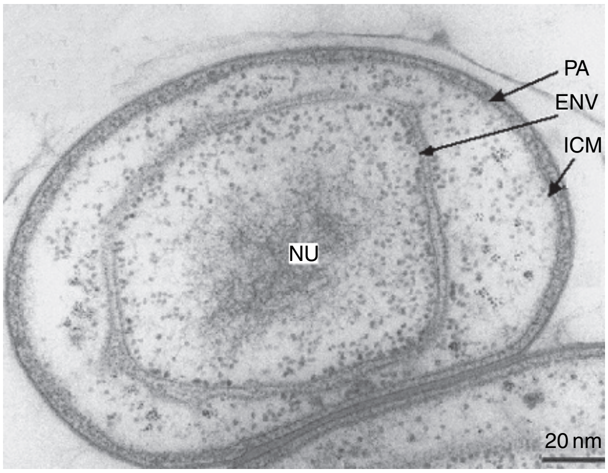

Figure 3 *Gemmata obscuriglobus*, a bacterium with an enveloped nucleoid. CM; ENV, envelope surrounding the nucleoid; ICM, intracytoplasmic membrane; NU, nucleoid; PA, paryphoplasm. Reproduced from Lindsay MR, Webb RI, Strous M, *et al.* (2001) Cell compartmentalisation in planctomycetes: Novel types of structural organisation for the bacterial cell. *Archives of Microbiology* 175: 413–429.

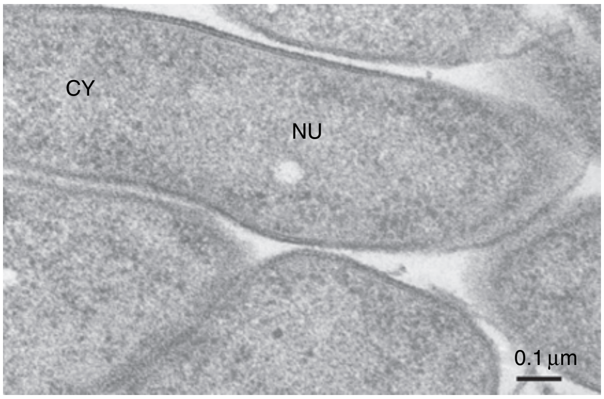

Figure 4 Electron micrograph of a thin section of *Halobacterium halobium*. The nucleoplasm in this archaeal organism is in direct contact with the cytoplasm. CY, cytoplasm; NU, nucleoplasmic region. Reproduced from Stoeckenius W and Rowen R (1967) A morphological study of *Halobacterium halobium* and its lysis in media of low salt concentration. *The Journal of Cell Biology* 34: 365–393.

cytoplasmic membrane is a ribosome-free (though not RNA-free) compartment termed paryphoplasm. Interestingly, compartmentalization of the nucleoid is reminiscent of the eukaryotic structure. Thus, posing the questions. What is the evolutionary position of the planctomycetes? Are they on their way to become eukaryotes? Did archaea become endosymbionts of planctomycetes? Did noncompartmentalized bacteria evolve into planctomycetes? Or, alternatively, did they lose compartments during evolution? In any case, the membrane-bounded nucleoid of *G. obscuriglobus* is quite different from a genuine nucleus, because its DNA is surrounded by cytoplasmic material. Clearly, there are many questions to be answered for the future. These novel data show an increasing diversity of bacterial ultrastructure.

Modern studies of Archaea have started about 20 years after those of Bacteria. For instance, seminal ultrastructural and biochemical works on the archaeon *Halobacterium halobium* originated in the mid-1970s of the previous century (**Figure 4**). This electron micrograph is perhaps the earliest image of an archaeon showing its prokaryotic nature, that is, the direct contact of the nucleoplasm and the cytoplasm. Habitats of archaea are diverse and may be harsh (hyperthermophilic or extreme halophilic) according to human standards. Up till now, no dominant model organism has emerged for the archaea. Nevertheless, the discovery that archaea are genetically more close to eukaryotes than bacteria makes them a highly interesting domain from an evolutionary point of view.

Shape of Prokaryotes

The shape of prokaryotes ranges from rods to spheres. Rods can be straight, curved, or helical. Cells may operate individually, as chains, when cell separation fails after division, as two-dimensional sheets, or even as three-dimensional packets. Cell shape is maintained by a rigid cell wall, which varies in complexity. The cell wall can be considered as an exoskeleton, which in the case of most bacterial species contains peptidoglycan. Archaea have no peptidoglycan and may contain pseudopeptidoglycan (pseudomurein) or chondroitin in some organisms. Consequently, archaea are not susceptible to antibiotics directed against the penicillin-binding proteins involved in peptidoglycan synthesis. Other archaea that lack pseudopeptidoglycan or chondroitin are bordered by a cytoplasmic membrane, which is reinforced by a so-called S-layer (see below).

In recent years, a new dimension has been added to the understanding of bacterial shape by the discovery in rod-shaped bacteria of a helix composed of a polymer of an actin-like protein MreB underneath the cytoplasmic membrane (**Figure 5**). In *E. coli* and *B. subtilis*, it runs from pole to pole. Disruption of the helix leads to spherical cells. This fits with the idea that shape involves interplay between cytoplasmic MreB and the envelope-associated peptidoglycan synthesizing system. Rod-shaped archaea may also posses MreB homologues, whereas coccal species tend to lack this protein. The helical shape of a Gram-negative spirochete like *Borrelia burgdorferi* is maintained by periplasmic flagella in addition to its peptidoglycan layer. Many prokaryotic species posses appendages such as flagella, pili (fimbriae), or a stalk (prosthaeca).

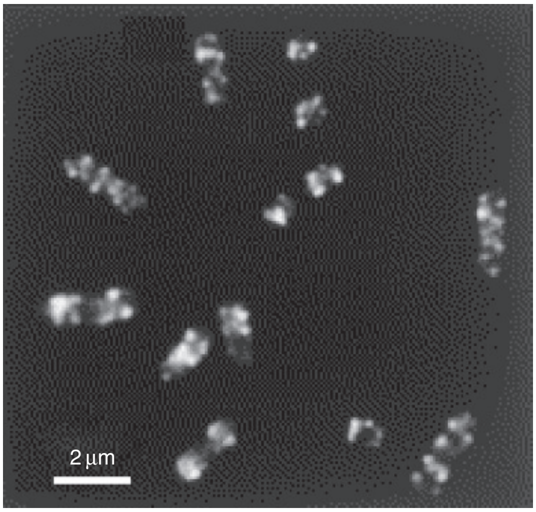

Figure 5 Fluorescent image of immunolabeled MreB in *Bacillus subtilis* 168. Reproduced from Jones LJF, Carballido-Lo′ pez R, and Errington J (2002) Control of cell shape in bacteria, helical, actin-like filaments in *Bacillus subtilis*. *Cell* 104: 913–922.

In presenting prokaryotic structure, I will proceed from the outside to the inside, that is, from cellular appendages to the nucleoid. In each section of this article, I will first focus on bacteria and subsequently, if applicable, on archaea.

Cell Envelope – Appendages

Prokaryotic cells often carry appendages such as a stalk, flagella, or pili. These serve to attach to surfaces (stalk), to allow swimming (flagella) or movement on a surface like swarming (flagella) or gliding (pili). Flagella and pili are distinct macromolecular complexes, whereas a stalk is a structural continuation of the cell envelope.

Stalks (Prosthecae)

Stalks enable cells to bind to a substrate. A familiar example is the stalk of *C. crescentus*. This organism is remarkable for its two alternating developmental stages where it exists as a polarly-flagellated free swimming cell, a swarmer or as a sessile stalked cell. When a swarmer sheds its flagellum and adjacent pili, a stalk develops at the same location. The stalk is much thinner than the diameter of the cell and it is free of cytoplasm. Thus, it basically represents a continuation of the bacterial envelope. Its increase in length bears resemblance to cell elongation as it takes place in *C. crescentus* and in *E. coli*. In these organisms, penicillin-binding protein 2 and RodA are required for cell elongation as well as growth of the stalk in *C. crescentus*. However, its length extension is not dispersed as in *E. coli*, it is rather carried out at its base, that is, at the junction of the stalk and the cell body.

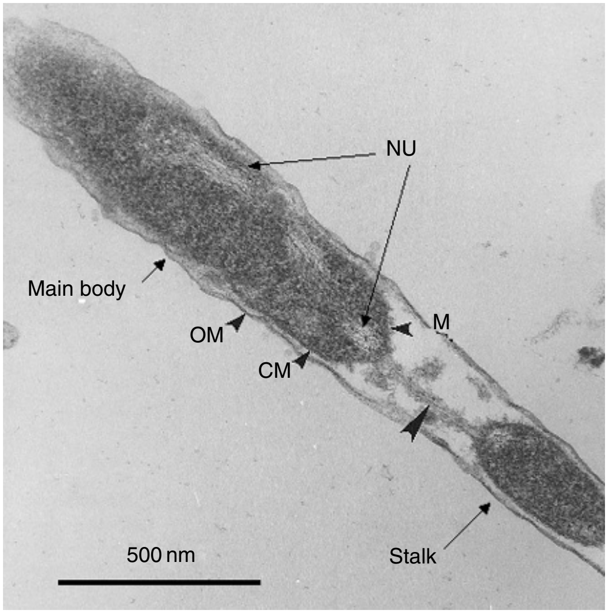

Figure 6 Electron micrograph of a thin section of *Hyphomonas* strain VP-6. CM, cytoplasmic membrane; M, membrane separating cytoplast from the stalk; NU, nucleoplasmic areas; OM, outer membrane. Arrow, membranous strands connecting cell and stalk contents. Reproduced from Zerfas PM, Kessel M, Quintero EJ, and Weiner RM (1997) Fine-structure evidence for cell membrane partitioning of the nucleoid and cytoplasm during bud formation in *Hyphomonas* species. *Journal of Bacteriology* 179: 148–156.

Hyphae (long filaments) are prosthecae that are seen in organisms such as *Hyphomonas* and *Hyphomicrobium*. Though *Hyphomonas* species and *C. crescentus* are quite similar genomically, their stalks serve essentially different purposes. In *Hyphomonas* species, the stalk is not devoid of cytoplasm (**Figure 6**). By contrast, it enables the migration of DNA and cytoplasm to the distal end of the stalk, where a bud is formed. The bud develops into a swarmer cell, which can be transformed into a stalked cell as in *C. crescentus*.

Bacterial Flagella

Many bacteria move by proteinaceous filaments, flagella, which are attached to their bodies. Bacterial flagella are hollow helical tubes with a length of several µms and a diameter of about 24 nm (**Figure 7**). The arrangement can be polar, peritrichous (peri, around; trichous, hair), or both. Polar flagella are longer than their peritrichous counterparts. Polarly flagellated *Spirillum volutans* has about 50 flagella at each pole. Spirochetes carry flagella in the periplasmic space between the outer membrane and the cytoplasmic membrane.

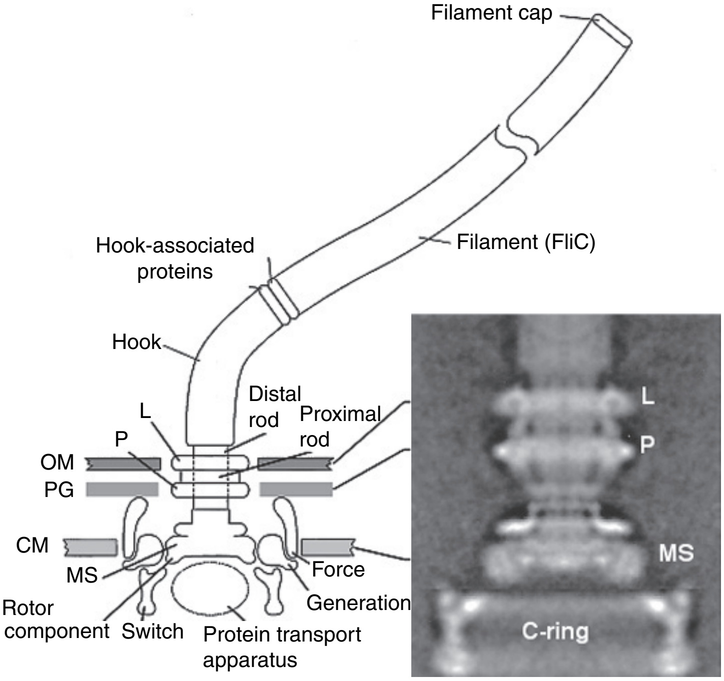

Figure 7 Schematic structure of a Gram-negative bacterial flagellum. At the right, an averaged image based on numerous electron micrographs. The C-ring is underneath the cytoplasmic membrane. CM, cytoplasmic membrane; L, L-ring in outer membrane; MS, MS-ring in cytoplasmic membrane; OM, outer membrane; P, P-ring in peptidoglycan layer; PG, peptidoglycan layer. Reproduced from Berg H (2003) The rotary motor of bacterial flagella. *Annual Review of Biochemistry* 72: 19–54.

The flagellum is anchored to the cell surface, where it is in contact with its various layers. Its integration into the envelope leads to various rings (MS, P, and L), specific for a particular layer, as indicated in **Figure 7**. The envelope-based structure is termed basal body. This structure as deduced from a rotationally averaged reconstruction of numerous electron micrographs is also shown in **Figure 7**. The flagellum is composed of three parts, the basal body with a rotator in the envelope, a bended flexible part called hook, and the more rigid filament. Flagellar assembly requires the participation of many different proteins. A protein export facility (type III secretion system) for flagellar proteins is apposed against the cytoplasmic side of the basal body. Elongation of the flagellar filament involves the transport of flagellin FliC from the cytoplasm, through the base of the flagellum and finally through a channel inside the flagellum to its distal part. Eleven protofilaments constitute the filament. The proteinaceous rotator (**Figure 7**) resembles a mechanical rotor in the sense that a cylinder can rotate in a stationary shaft, the stator. The energy for torque generation is thought to result from the proton motive force across the cytoplasmic membrane.

In Gram-negatives such as *E. coli* and *S. typhimurium*, flagella may rotate clockwise or counterclockwise. In the first case, cells show directionless tumbling, whereas in the other, they swim in a straight line. Tumbling arises when intertwined flagella during counterclockwise rotation change their helical pitch. The alternation between tumbling and straight swimming makes it possible to adhere to a directed movement when a food source (attractant) or repellent has been located. Sensing of the food source occurs through chemoreceptors located near the cellular pole. A signal transduction system provides a connection with the basal body of the flagellum. The flagella of Gram-positives resemble those of Gram-negatives, but their integration in the cell envelope reflects the difference in cell wall structure of the two types of microorganisms. A remarkable feature characterizes spirochetes, for instance, in *Borella* species and *Treponema* species. In these organisms, periplasmic flagella arise at a subpolar position from each pole and overlap in the cell center. Presumably, because of their location, the flagella are directly responsible for the helical shape of the spirochetes and indirectly for the motility of the latter.

Archaeal Flagella

Archaeal flagella, as studied in the extreme halophile *Halobacterium salinarium*, are quite different from those of bacteria. They have smaller dimensions than their bacterial equivalents and their axial filaments contain different proteins. The diameter of archaeal flagella is about 10 nm, whereas it is 24 nm for the bacterial organelles (**Figure 8**). Another difference is that their proteins are often

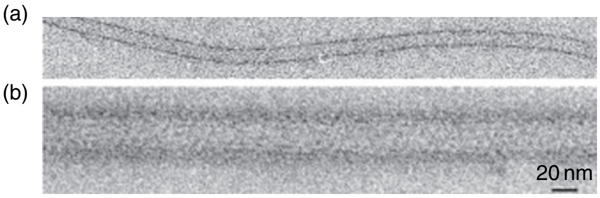

Figure 8 Electron micrographs of negatively stained bacterial and archaeal flagella allowing diameter comparison. (a) An archaeal flagellum. (b) A flagellum of *Salmonella typhimurium*. Reproduced from Cohen-Krausz S and Trachtenberg S (2002) The structure of the archaebacterial flagellar filament of the extreme halophile *Halobacterium salinarum* R1M1 and its relation to eubacterial flagellar filaments and type IV pili. *Journal of Molecular Biology* 321: 383–395.

glycosylated. Presumably, this is related to the requirement to sustain extreme growth conditions, be it high salinity or high temperature. Note that probably for the same reason, S-layers of archaea are also glycosylated. The architecture of archaeal flagella bears resemblance to that of type IV pili (**Figure 9**). Likewise, the N-termini of archaeal flagellin and bacterial pilin show significant homology. Knowledge about archaeal flagella is emerging slowly. In the hyperthermophile *Pyrococcus furiosus*, which grows at 100 °C, a bundle of flagella is inserted at one pole. Their glycosylated proteins resemble those of other archaeal flagella. The *P. furiosus* flagella adhere to surfaces, and it remains to be demonstrated whether they are used for swimming. Clearly, research on archaeal flagella requires continued efforts.

In contrast to bacterial flagella, the growth of archaeal flagella might take place at their base because they seem to lack a central channel. However, the detailed interactions of cell-proximal archaeal proteins with the cell envelope are not yet known. In *H. salinarium*, the flagellum is thought to interact with a cytoplasmic structure underneath the cytoplasmic membrane at a cell pole. This structure has been termed polar cap (**Figures 9(b)** and **10**). So far, it is not clear whether a cell envelope-based rotor is part of an archaeal flagellum. Bio-assembly of the archaeal flagellum is thought to occur via a type II secretion system as in type IV pili.

Pili (Fimbriae)

Pili are comparatively small rod-like proteinaceous appendages on bacterial surfaces. They are markedly smaller

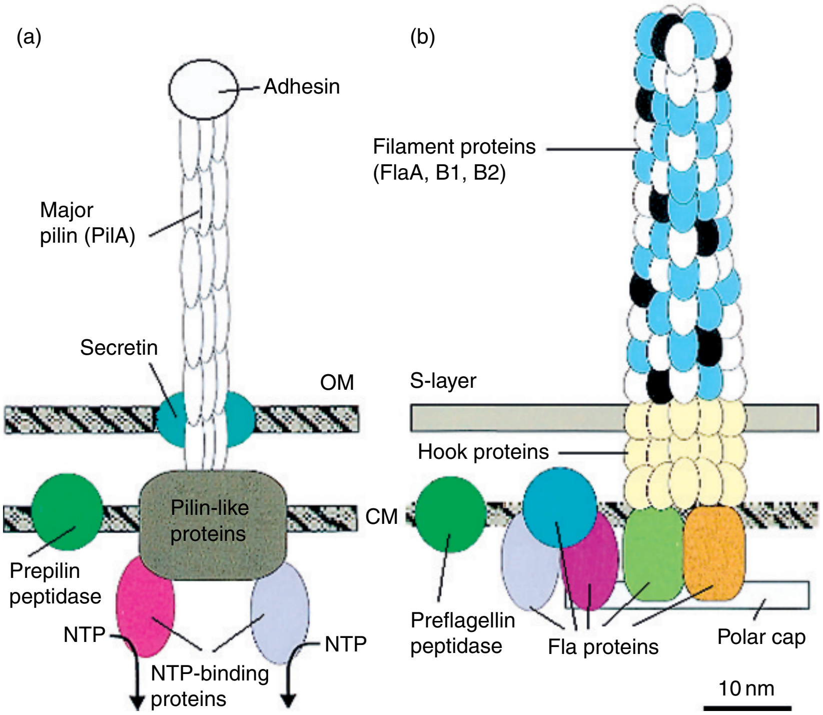

Figure 9 Schemes of the possible architecture of a (a) type IV pilus and an (b) archaeal polar flagellum. (a) The main body from the type IV pilus is composed of the pilin protein PilA. Pilin-like proteins are cleaved by the prepillin peptidase in the cytoplasmic membrane (CM). The secretin allows protrusion of the pilus through the outer membrane (OM). The energy for assembly and retraction of the pilus is supposed to be delivered by the nucleotide triphosphate (NTP)-binding proteins. (b) The external part of the flagellum is made up of the three flagellar proteins FlaA, FlaB1, and FlaB2. The flagellum is probably embedded in the envelope with other Fla proteins. A preflagellin peptidase cleaves the leader peptide from flagellins to be incorporated into the flagellum. Not all functions of proteins are known. CM, cytoplasmic membrane; OM, outer membrane; PC, polar cap (cf. **Figure 10**); SL, S-layer. Reproduced from Bardy SL, Ng SYM, and Jarrell KF (2003) Prokaryotic motility structures. *Microbiology* 149: 295–304.

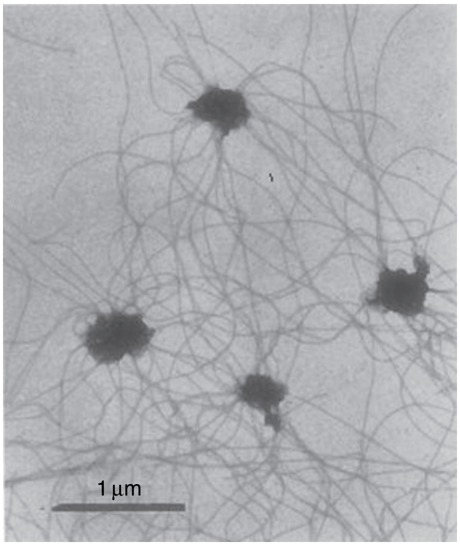

Figure 10 Electron micrograph of polar caps of *Halobacterium halobium* purified by gel filtration. Reproduced from Kupper J, Marwan W, Typke D, *et al*. (1994) The flagellar bundle of *Halobacterium salinarum* is inserted into a distinct polar cap structure. *Journal of Bacteriology* 176: 5184–5187.

than flagella. Information on archaeal 'pili' is slowly emerging (see below). The terms pili and fimbriae are both used, mostly for one and the same thing. Examples include P pili, type I pili, type IV pili, and G fimbriae. In this section, the focus is on type I and type IV pili, because they have been studied extensively. At their tips, pili carry adhesive proteins. Nonfimbrial adhesive proteins (adhesins) may also be present directly on the bacterial surface.

Type I pili

These pili, which resemble P pili, emerge from the surface of *Enterobacteriaceae*. The pili are somewhat rigid thin filaments, which can attain a length of 2 μm (**Figure 11**). In uropathogenic *E. coli* strains, they adhere to the surface of epithelial cells with their tips. At the tip, the adhesin FimH is present, which is a mannose-specific lectin. The main body of type I pili is composed of FimA subunits, which are arranged into a helix via three protofilaments (**Figure 12**). The pilus has a diameter of 6–7 nm and an axial hole with a diameter of 2.0–2.5 nm. Bio-assembly of type I pili is carried out through the chaperone-usher pathway.

Type IV pili

These pili reside on polar surface of pathogenic Gram-negative bacteria, such as *Neisseria gonorrhoae, Neisseria meningitidis*, and the opportunistic pathogen *Pseudomonas aeruginosa*. They are multifunctional, including acting in adhesion, uptake of DNA (natural transformation), twitching motility, and biofilm formation. Adhesion and DNA uptake are mediated at the pili tips. Type IV pili have a length in the order of 1 to several micrometers and a thickness of about 5 nm. The pili have a helical structure and contain as a main component PilA (**Figure 9(a)**). Probably related to their function, type IV pili can retract and extend. This is supposed to enable gliding by twitching motility. The formation of fruiting bodies by directed movement of *Myxococcus xanthus* is termed social gliding and it also involves twitching motility. Swarming, as it occurs in *Proteus* sp., can also be considered as a form of social gliding. However, it involves flagella and not pili. Bio-assembly of type IV pili is mediated by the type II secretion system.

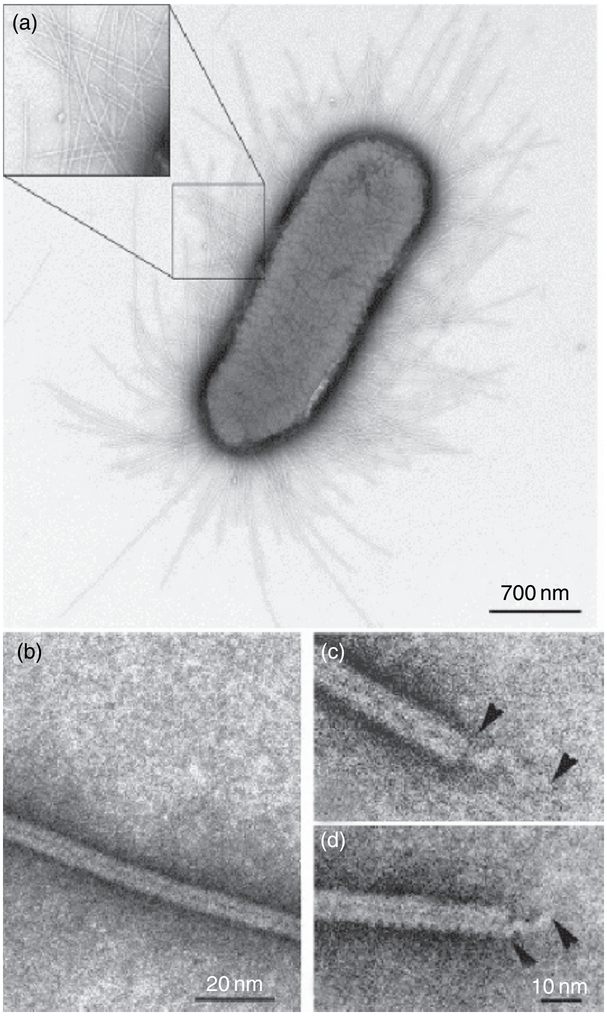

Figure 11 Electron micrographs of *Escherichia coli* W3110 and type I pili. (a) Negatively stained cell with pili. Inset: detail at higher magnification. (b) Darkfield image of a pilus. The pilus seems to have a channel as suggested by its dark central line. (c, d) Darkfield image of pili extremities. Arrows point to a presumed flexible coiled tip. Reproduced from Hahn E, Wild P, Hermanns U, *et al*. (2002) Exploring the 3D molecular architecture of *Escherichia coli* type 1 pili. *Journal of Molecular Biology* 323: 845–857.

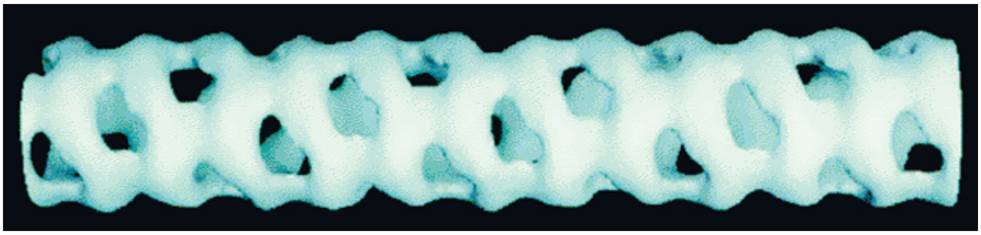

Figure 12 A 3D reconstruction of type I pilus composed of FimA. Reproduced from Hahn E, Wild P, Hermanns U, *et al*. (2002) Exploring the 3D molecular architecture of *Escherichia coli* type 1 pili. *Journal of Molecular Biology* 323: 845–857.

Hami

Bacterial pili and flagella have been studied extensively. By contrast, much less is known about archaeal flagella (see above) and even less about potential archaeal pili equivalents. Hami are a novel type of appendages that are peritrichously arranged on the surface of a nonmethanogenic archaeal organism denoted SM1 (**Figure 13**). Their name is derived from the Latin term *hamus*, which denotes prickle or hook amongst others. They are several microns long and have a diameter of 7–8 nm. Remarkably, their tips end in a structure resembling a triple fishhook (**Figure 14**). The fishhooks are preceded by a smooth region, which, in turn, is preceded by the remaining filament region. This region that extends to the cellular surface resembles barbed wire (**Figure 14**). The barbed wire appearance is caused by the periodic arrangement of three prickly structures along the helical filament. The latter is presumed to be composed of three protofilaments. The detailed dimensions are also given in **Figure 14**. So far, nothing is known about the assembly pathway of these elegant structures. It is in particular intriguing to understand how the fishhook-like structures are created. Hami have adhesive properties. The tip structures suggest that this is accomplished through grasping rather than through stickiness as in adhesive proteins.

Archaeal pili of known chemical composition have not been identified morphologically. However, they might be present, because an archaeal genome search for bacterial pilin-like and pilin-associated proteins has identified several components that point to the existence of class III signal peptides. It should be remembered that bacterial

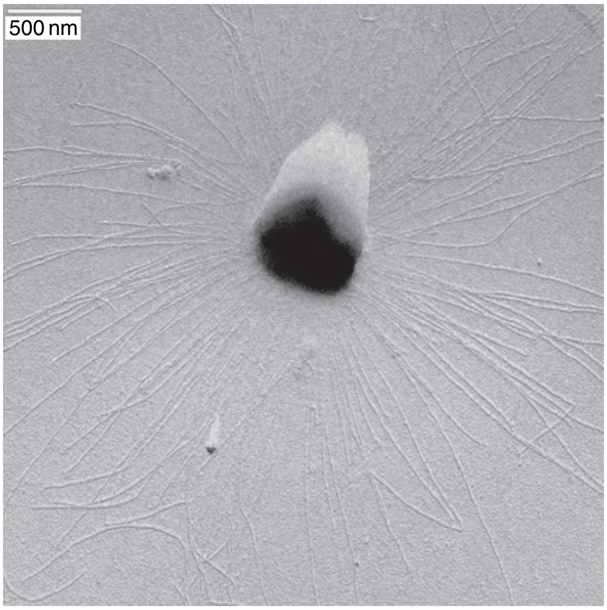

Figure 13 Electron micrograph of a shadowed archaeal coccus SM1 with hami on its surface. Reproduced from Moissl C, Rachel R, Briegel A, Engelhardt H, and Huber R (2005) The unique structure of archaeal 'hami', highly complex cell appendages with nano-grappling hooks. *Molecular Microbiology* 56: 361–370.

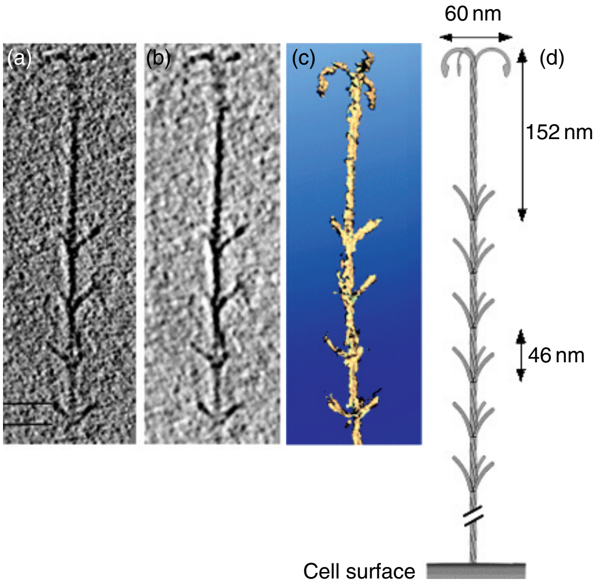

Figure 14 Detailed representation and model of a hamus based on cryo-electron tomography. (a) Digital section through a 3D reconstruction of a hamus. (b) After denoising. (c) Surface rendering of the data from Figure 14b. (d) Detailed dimensions of hamus structure. Reproduced from Moissl C, Rachel R, Briegel A, Engelhardt H, and Huber R (2005) The unique structure of archaeal 'hami', highly complex cell appendages with nano-grappling hooks. *Molecular Microbiology* 56: 361–370.

type IV pili also have class III signal peptides. Clearly, for the future, archaea still have much in store concerning their cellular appendages.

Cell Envelope

The cell wall functions to protect the integrity of the cell and at the same time it permits interaction of the organism with a variable environment. The Gram-negative cell envelope as exemplified in *E. coli* (**Figure 2**) is composed of three layers. From outside to inward, these are the outer membrane, the peptidoglycan layer, and the inner or cytoplasmic membrane. The peptidoglycan layer is covalently attached to the outer membrane through lipoprotein molecules. This is easily seen upon plasmolysis, when outer membrane and peptidoglycan layer peel away together from the cytoplasmic membrane. The compartment between the two membranes and which includes the peptidoglycan layer is called the periplasm. It is filled with membrane-derived oligosaccharides. Their synthesis is under osmoregulation and they are thought to function in maintaining a high osmotic pressure in the periplasm.

The Gram-positive cell lacks an outer membrane; instead, it has a rather thick cell wall (**Figure 2**) composed of peptidoglycan and wall teichoic acids. Wall teichoic acids are charged anionic polyol phosphates, which give a negative charge to the Gram-positive cell wall. Gram-positive bacteria are difficult to plasmolyze, presumably because the molecular interactions between cytoplasmic membrane and cell wall are relatively abundant. For instance, lipoteichoic acid polymers are inserted into the cytoplasmic membrane, thus spanning the whole envelope. A few organisms such as *Mycoplasma, Planctomyces*, and *Chlamydia* lack peptidoglycan.

Archaea can also be divided into Gram-positives and Gram-negatives, though this distinction is not as widely used as for bacteria. Archaea lack peptidoglycan, instead they may possess pseudopeptidoglycan (pseudomurein) in their walls. Other archaea contain a chondroitin-like polymer. In some wall-less organisms, the cytoplasmic membrane is reinforced by an S-layer (see below). In the hyperthermophilic *Ignicoccus* sp., an outer membrane has been detected. This structure should not be confused with the outer membrane of *E. coli*. Still other archaea may lack a cell wall, without a reinforcing S-layer, which results in a flexible shape.

In Gram-positive as well as Gram-negative organisms, an additional external layer, composed of regularly arranged proteins may be present (S-layer; see below). S-layers, as already mentioned, also occur in archaea. In some archaea, they are in direct contact with the cytoplasmic membrane and contribute to maintaining the cell shape (see below).

Capsules

Long polysaccharides associated with the outer membrane of, for instance, *E. coli* protrude in the environment and together form a capsule at the surface of the cell (**Figure 2**). The polysaccharides are extremely variable in composition and are strain-specific. In *E. coli*, K antigens and O antigens are distinguished.

S-Layers

The disposition of the S-layer on the envelopes of bacteria and archaea is shown in **Figures 15(a)** and **16**. In Gram-negative archaea such as *H. halobium*, S-layer

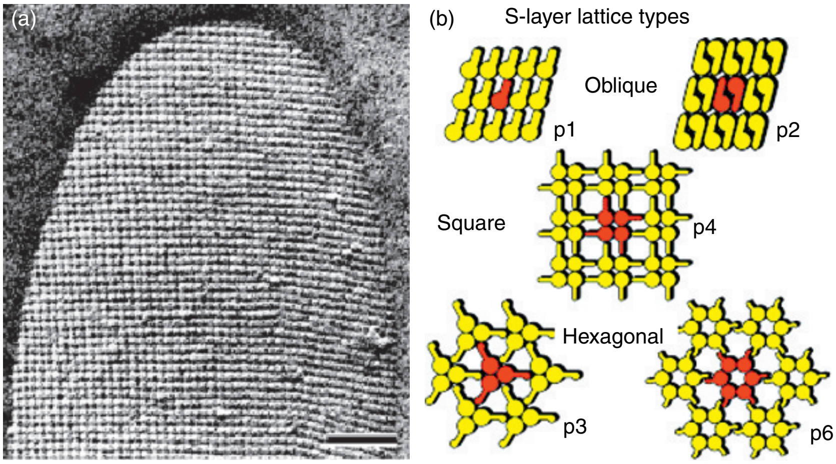

Figure 15 Electron micrograph of a freeze-etched cell surface and different S-layer lattice types. (a) Square lattice on the outer surface of *Bacillus sphaericus* CCM 2177. (b) Oblique (p1, p2), square (p4), and hexogonal (p3, p6) lattice symmetries. The numbers refer to the identical morphological units indicated in red. Bar: 100 nm. Reproduced from Sleytr UB, Huber C, Ilk N, Pum D, Schuster B, and Egelseer EM (2007) S-layers as a tool kit for nanobiotechnological applications. *FEMS Microbiology Letters* 267: 131–144.

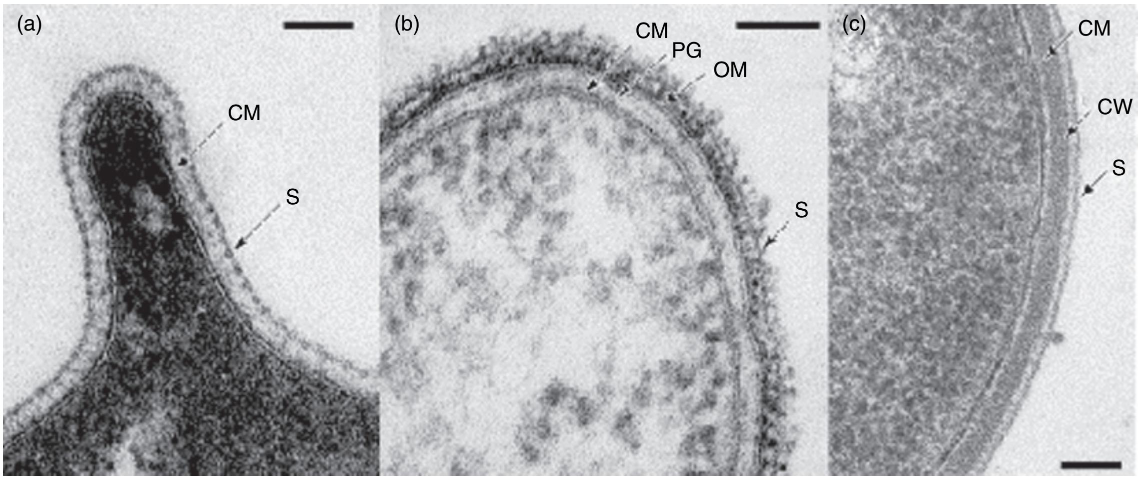

Figure 16 Electron micrographs of thin sections of diverse cell envelopes with S-layers (cf. **Figure 17**). (a) The S-layer (S) apposed to the cytoplasmic membrane (CM) in *Sulfolobus acidocaldarius*. (b) S-layer apposed to the outer membrane (OM) of the Gram-negative *Aeromonas salmonicida*. (c) The S-layer on the cell wall (CW) of the Gram-positive *Bacillus thuringiensis*. PG, peptidoglycan layer. Bar: 50 nm. Reproduced from Sleytr UB and Beveridge T (1999) Bacterial S-layers. *Trends in Microbiology* 7: 253–260.

(glyco)proteins are inserted into the outer leaflet of the inner membrane (**Figure 17(a)**). In Gram-positive bacteria and Gram-positive archaea, the S-layer appears apposed to the cell wall containing peptidoglycan or pseudopeptidoglycan, respectively (**Figure 17(b)**). In Gram-negative bacteria, the S-layer is in contact with lipopolysaccharide (LPS) constituent of the outer membrane (**Figure 17(c)**). S-layers arise through self-assembly on the cells' surface and they might obey different rules of symmetry (**Figure 15(b)**). Purified S-layer proteins assemble into sheets *in vitro*. The S-layer may disappear under laboratory growth conditions. S-layers have likely a protective function, one aspect of which might be a role as a molecular sieve. Nanobiotechnological applications of S-layer proteins include their crystallization on specific substrates. This in turn can be used to organize selected biomaterials in a defined pattern.

Outer Membrane

The outer membrane is an integral component of the cell envelope of Gram-negative bacteria, where it is thought to act as a selective permeability barrier mainly. It is composed of (lipo)proteins, phospholipids, and LPSs. The arrangement of these components in the outer membrane is essentially asymmetric (**Figure 17(c)**). The chemical asymmetry is clearly seen in freeze-fractured membranes at the ultrastructural level.

The LPSs are located in the outer leaflet of the outer membrane, whereas the main phospholipids reside in the inner leaflet (i.e., the leaflet pointing to the interior of the cell). LPS consists of three regions: lipid A, which is anchored to the outer leaflet of the outer membrane, core oligosaccharide, and O-specific polysaccharides, also called O-antigen. The composition of the latter can be extremely variable and the genes that express O-antigen are grouped in gene clusters.

Outer membrane proteins tend to be organized into trimers to allow their function as hydrophilic transmembrane channels. As such, the channel proteins are referred to as porins. Several porins may occur in one and the same cell and they have been studied in many Gram-negative organisms. A monomeric porin has a ß-barrel structure, which traverses the outer membrane. Three ß-barrel structures constitute a pore as shown for the osmoporin OmpC (**Figure 18**).

Peptidoglycan Layer

Escherichia coli

Peptidoglycan or murein is constructed from glycan chains interconnected by peptide side chains. The glycan chains consist of disaccharide subunits composed of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). They are connected through ß(1,4) glycosidic bonds. Their length is extremely variable and can range from 8 to 80 disaccharide units. The glycan chains carry peptide side chains (stem peptides), which can be interconnected through peptide bonds. In *E. coli*, the side chains are tetrapeptides, which are interconnected through a peptide bond between *meso*-diaminopimelic acid and D-alanine (**Figure 19(a)**). Whereas the composition of the glycan chains is quite universal, the composition of the peptide side chains may differ in various organisms. For instance, in a Gram-positive organism like *S. aureus*, the peptide is a pentaglycine. A consequence of peptide side chain cross-linking is that the peptidoglycan layer represents a single covalent structure, which has the shape of the cell (**Figure 20**). In a Gram-negative organism like *E. coli*, the peptidoglycan

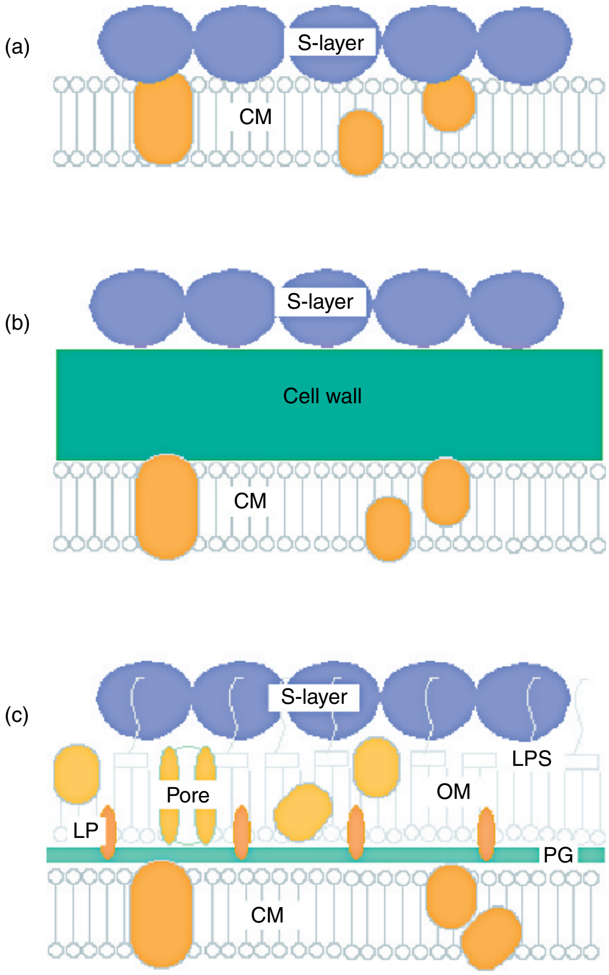

Figure 17 Schematic disposition of S-layers in archaea and bacteria. (a) S-layer on the cytoplasmic membrane (CM) of an archaeon (cf. **Figure 16** (**a**)). (b) A Gram-positive archaeon or bacterium. Cell walls have different chemical compositions in the two cases. (c) S-layer associated with the lipopolysaccharide (LPS) leaflet of the outer membrane (OM) in a Gram-negative bacterium. LP, lipoprotein; PG, peptidoglycan layer. Reproduced from Sleytr UB and Beveridge T (1999) Bacterial S-layers. *Trends in Microbiology* 7: 253–260.

layer is monomolecular and thus very thin. This singular sac-like macromolecule has been called a *sacculus* or little sac. The position of the glycan chains in the plane of the peptidoglycan layer is not known for certain, though there is evidence indicating that they are arranged more or less perpendicular to the length axis of the cell. The sacculus is not a static structure, because it is subject to turnover and recycling of its constituents.

Assembly of the peptidoglycan layer in *E. coli* takes place in three cellular compartments. A series of enzymatic reactions in the cytoplasm produces UDP-MurNAc-pentapeptide. The next step takes place at the cytoplasmic membrane where lipid I and lipid II are formed subsequently. Lipid I is the result of binding UDP-MurNAc-pentapeptide to undecaprenyl phosphate. The addition of UDP-GLcNAc to lipid I produces lipid II. The third compartment is the periplasm, where lipid II is inserted into existing peptidoglycan by penicillin binding-proteins. To achieve this, the prenylated disaccharide pentapeptide has to switch from the cytoplasmic side of the membrane to the periplasmic side by an unknown flippase activity.

Bacillus subtilis

In the Gram-positives, the peptidoglycan-containing walls are much thicker than their Gram-negative counterparts. Moreover, wall teichoic acids are intermeshed and covalently linked to peptidoglycan. The molecular spatial architecture of the Gram-positive cell wall is not well-known. Recently, an interesting comparison has been made between freeze-substituted and frozen-hydrated electron microscopic sections of *B. subtilis* strain 168 (**Figures 21**(**a**) and **21**(**b**)). Freeze-substitution involves rapid freezing to preserve ultrastructure. Cells are then fixed, dehydrated, and stained at low temperature (−80 °C). This procedure is followed by classical embedding to enable thin sectioning. Clearly,

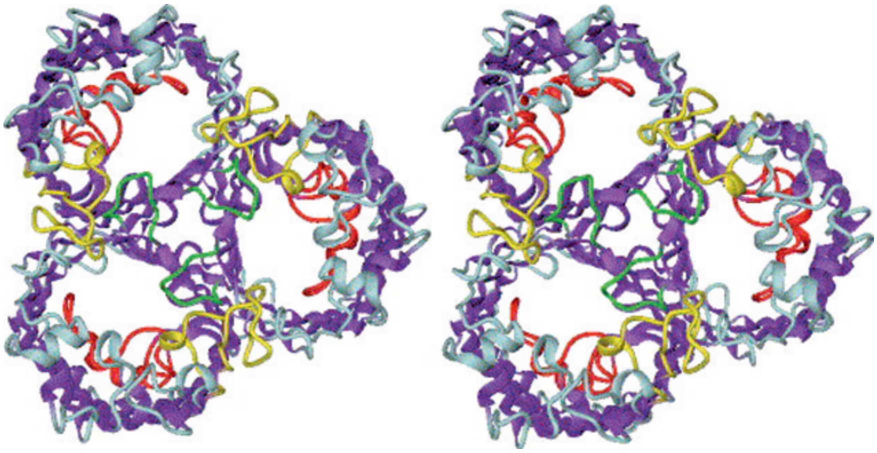

Figure 18 Stereo representation of the OmpC trimer structure as viewed from the extracellular side. Reproduced from Basle A, Rummel G, Storici P, Rosenbusch JP, and Schirmer T (2006) Crystal structure of osmoporin OmpC from *E. coli* at 2.0A. *Journal of Molecular Biology* 362: 933–942.

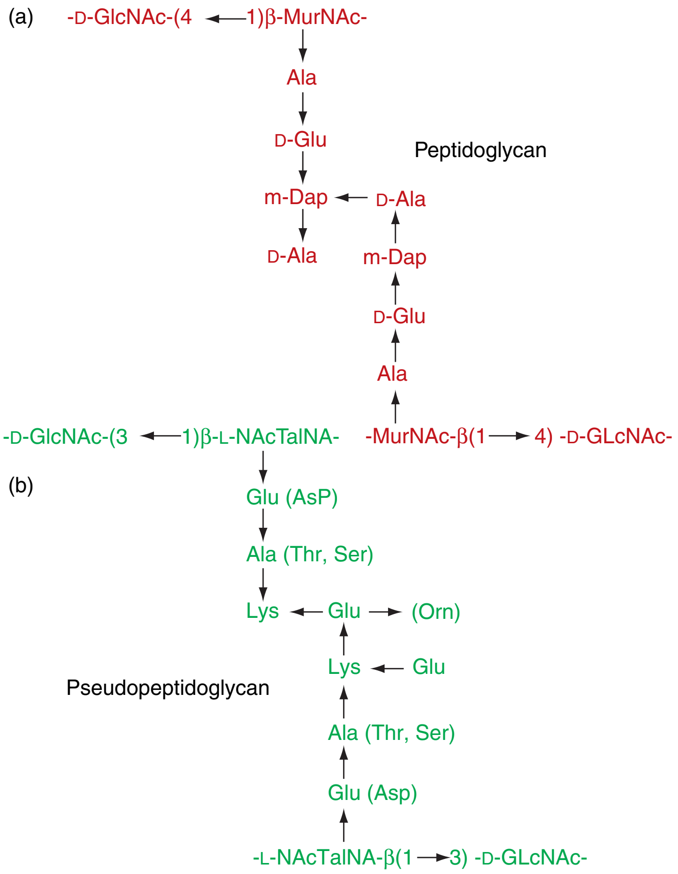

Figure 19 Schematic structure of peptidoglycan and pseudopeptidoglycan. Note that pseudopeptidoglycan does not contain D-amino acids.

dehydration is a critical step in this procedure, though perhaps less so as at room temperature procedures. The cell wall image (**Figure 21(a)**) shows an electron dense region (1), a more translucent zone (2), and finally a ruffled electron dense outer layer (3). The tripartite cytoplasmic membrane is not distinguishable, presumably because the membrane and associated material has collapsed into a thin layer during dehydration. The interpretation of zone 2 is more difficult in this image. However, the ruffled outer zone fits with the concept that older peptidoglycan becomes dissolved by autolysins as it arrives at the wall surface. By contrast, frozen-hydrated sections do not require dehydration (by definition) and staining by electron-dense chemicals. Here, a contrast is produced through native differences in electron density. As seen in **Figure 21(b)**, the cytoplasmic membrane is well visible and the cell wall appears to be divided into two zones, an inner wall zone (IWZ) of relatively low electron density and an outer wall zone (OWZ) of higher electron density. Because the IWZ is absent in isolated cell walls, this has led to the idea that this low electron density zone represents the periplasm of *B. subtilis*. This is a novel concept for a Gram-positive like *B. subtilis* and it seems plausible because excreted proteins and penicillin-binding proteins need a space to carry out their tasks. Interestingly, the OWZ decreases in density from inside to outside. This implicates that the ruffled outer zone visible after freeze-substitution (**Figure 21(a)**) is the result of dehydration.

Assembly of peptidoglycan in *B. subtilis* is essentially the same as in *E. coli*. However, it is coordinated with the synthesis of wall teichoic acids. The coordination is achieved through the use of a common component – undecaprenyl phosphate. Remarkably, wall teichoic acids are assembled underneath the cytoplasmic membrane and not in the periplasm as for peptidoglycan. Ultimately, the wall teichoic acid polymer has to be transferred to the periplasm where it becomes attached to peptidoglycan. The transfer mechanism is not yet understood.

Peptidoglycan synthesis is different in cocci and rods in the sense that in cocci the cell wall synthetic activities are linked to septation.

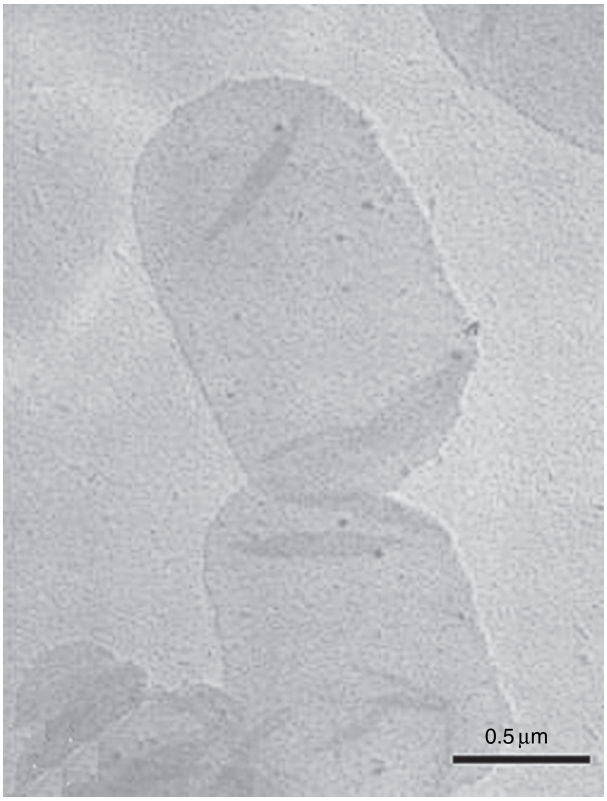

Figure 20 Electron micrograph of shadowed *sacculi* purified from *Escherichia coli.* Reproduced from Verwer RW, Nanninga N, Keck W, and Schwarz U (1978) Arrangement of glycan chains in the *sacculus of Echerichia coli*. *Journal of Bacteriology* 136: 723–729.

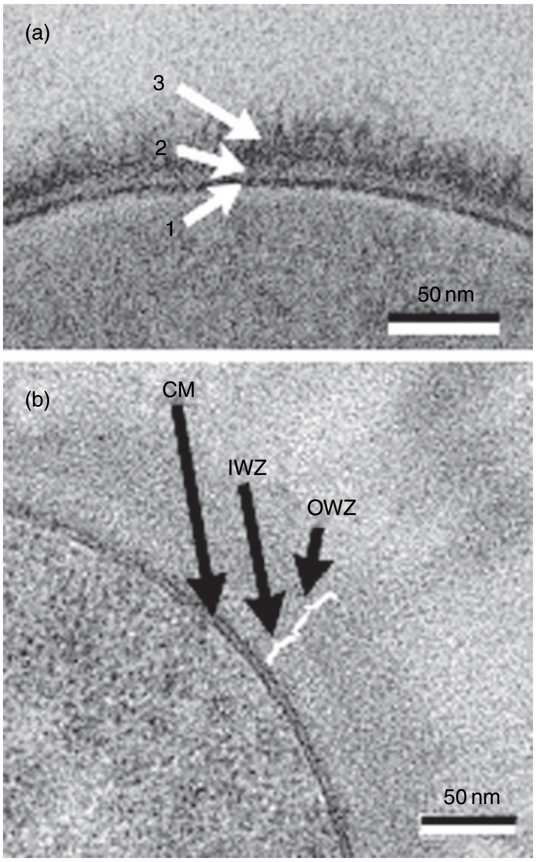

Figure 21 Electron micrographs of thin sections of freeze-substituted and frozen-hydrated *Bacillus subtilis* 168 cell envelopes. (a) Freeze-substituted image. 1, heavily stained innermost region, including cytoplasmic membrane; 2, intermediate region; 3, fibrous outermost wall region. (b) Frozen-hydrated image. CM, cytoplasmic membrane; IWZ, low density inner wall zone; OWZ, high density outer wall zone. Reproduced from Matias VRF and Beveridge TJ (2005) Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. *Molecular Microbiology* 56: 240–241.

Pseudopeptidoglycan

In some Gram-positive Archaea, the genus *Methanobacterium*, the cell wall contains pseudopeptidoglycan. In this polymer, the disaccharides are composed of GlcNAc and *N*-acetyltalosaminuronic acid (NacTalNA), where the latter has replaced bacterial MurNAc. Moreover, they are connected through a ß(1,3) glycosidic bond (**Figure 19(b)**). The glycan chains carry peptide side chains as in peptidoglycan. However, they lack D-amino acids. Assembly of pseudopeptidoglycan requires undecaprenyl phosphate as in peptidoglycan and teichoic acid synthesis.

Overall Structure

The cytoplasmic membrane as a phospholipid bilayer envelopes the cytoplasm. Though, by definition, a structural entity, its protein components interact functionally with cytoplasmic and periplasmic proteins. Thus, despite classic electron microscopic images of thin-sectioned bacteria, the cytoplasmic membrane is embedded in a proteinaceous framework. In recent years, fluorescence microscopy has revealed that proteins in or at the cytoplasmic membrane are positioned in a helical arrangement. A pioneering achievement has been the discovery of the MreB helix underneath the cytoplasmic membrane (**Figure 5**). Subsequently, it has been shown that, for instance, the Sec protein translocation machinery is helically arranged at the cytoplasmic membrane (**Figure 22**) in *B. subtilis* as well as in *E. coli.* In both cases, the Sec helix and the MreB helix do not overlap. Also membrane proteins with periplasmic domains may be organized into a helix. These findings stipulate the highly organized disposition of the cytoplasmic membrane in relation to periplasm and cytoplasm.

In some cases, membranous invaginations arise from the cytoplasmic membrane, for instance, in prototrophic, nitrifying, and methanotrophic bacteria. An outdated example is the mesosome in Gram-positive organisms,

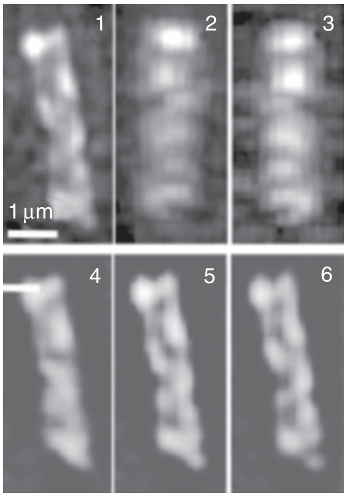

Figure 22 Fluorescent micrographs of the helical arrangement of a GFP-SecE fusion protein in *Escherichia coli* HCB436 cells. Panels 1 to 3, processed 3D-reconstituted images seen from different angles. Panels 4–6, unprocessed 3D-reconstituted images seen from different angles. Reproduced from Shiomi D, Yoshimoto M, Homma M, and Kawagishi I (2006) Helical distribution of the bacterial chemoreceptor via colocalization with the Sec protein translocation machinery. *Molecular Microbiology* 60, 894–906.

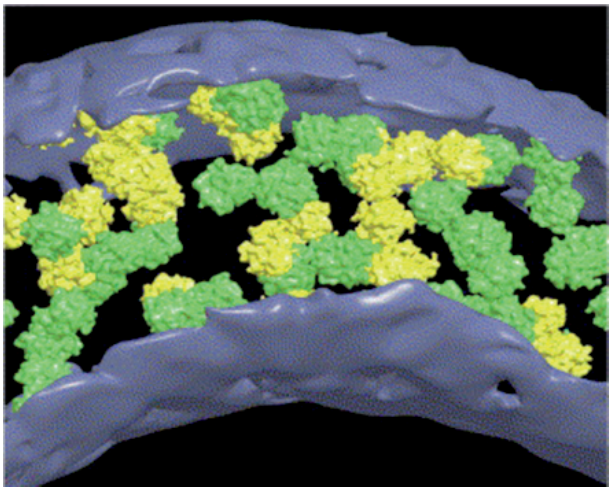

Figure 23 Arrangement of 70S ribosome in the cytoplasm of *Spiroplasma melliferum*. The ribosomes have been identified on the basis of pattern recognition and template matching using cryo-electron tomography. The identification of the green ribosomes is more reliable than the yellow ones from a statistical point of view. Grey structure: envelope. Reproduced from Ortiz JO, Förster F, Kürner J, Linaroudis A, and Baumeister W (2006) Mapping 70S ribosomes in intact cells by cryoelectron tomography and pattern recognition. *Journal of Structural Biology* 156: 334–341.

which is nowadays considered as an artifact of electron microscopic preparation.

In archaeal membrane lipids, hydrophobic side chains are not linked to the glycerol backbone through an ester linkage, and an ether linkage is used instead. Furthermore, the hydrophobic side chains are not fatty acids, but isoprenoid chains. These and other differences testify to the special status of archaea within the prokaryotes.

Cytoplasm

The Cytoplasm

The structural connection between transcription and translation leads literally to the picture of polyribosomes being attached to DNA (**Figure 1**). At a first approximation, the bacterial cytoplasm (at least in fast growing cells) can be conceived of as a compartment filled with polyribosomes. In electron microscopic thin sections, individual ribosomes are visible as ill-defined black dots because of severe dehydration, which precludes analysis of their spatial configuration. However, recent advances in cryo-electron tomography in combination with pattern recognition techniques have identified 70S ribosomes as such in a bacterial cell (*Spiroplasma melliferum*). This has opened the possibility to generate what has been termed a cellular atlas of macromolecular complexes (**Figure 23**). It was also observed that in *S. melliferum*, ribosomes comprise a limited fraction of the cytoplasmic volume.

As noted before, polyribosomes may link DNA and cytoplasmic membrane (**Figure 1**). In this sense, there is a transient structural link between nucleoplasm and envelope. It is not clear whether the collection of polyribosomes could serve as an intracellular supportive structure or even whether such a supportive structure would be needed at all. Ubiquitous cytoplasmic proteins, such as the ribosomal elongation factor Tu and the tubulin-like cell division protein FtsZ, are able to polymerize into linear polymers *in vitro*. Whether such polymers provide for a cytoplasmic framework *in vivo* is not clear (cf. **Figure 24**). As mentioned earlier, helical MreB polymers are located underneath the cytoplasmic membrane (see, however, below).

Some organisms contain polyhydroxybutyric acid granules, polyphosphates, sulfur droplets, or even magnetosomes. Gas vesicles are a special case, serving to affect the buoyant density of, for instance, *H. halobium*.

Cytoplasmic Proteinaceous Filaments

Eukaryotic cytoplasm is crammed with proteinaceous filaments. These constitute the cytoskeleton, wherein actin filaments and microtubules are ubiquitously present. Protein constituents of these structures are actin and tubulin, respectively. Filamentous structures composed of similar proteins also occur in bacterial cytoplasm.

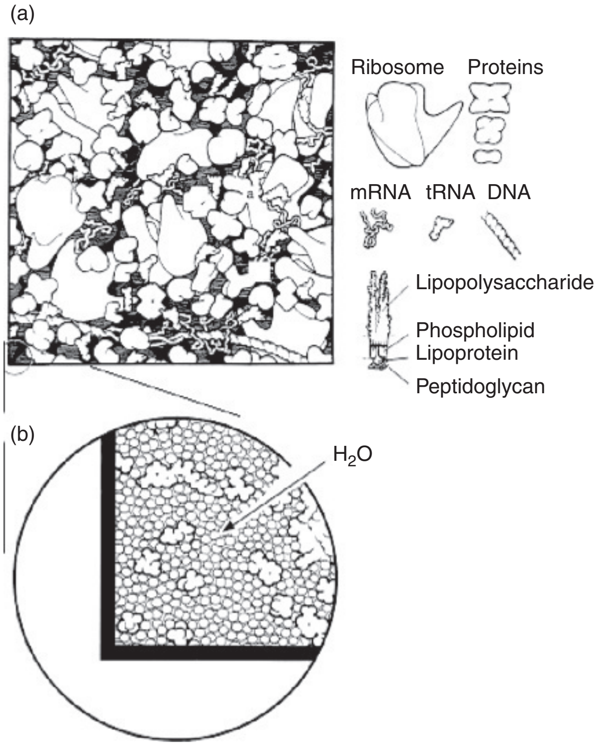

Figure 24 (a) A 100 nm window of the *Escherichia coli* cytoplasm. Ribosomes and other components have been drawn to scale. (b) A close-up of part of the window as indicated. Depicted are water molecules, some larger molecules, and part of a protein. Reproduced from Goodsell DS (1991) Inside a living cell. *Trends in Biochemical Sciences* 16: 203–206.

However, their spatial disposition is essentially different. In a previous section, mention has already been made of the actin-like protein MreB, the polymers of which form a helix underneath the cytoplasmic membrane (cf. **Figure 5**). Prokaryotes also contain a tubulin homologue, which is called FtsZ. Upon division, FtsZ polymerizes in the cell center into a ring-like structure, which is apposed against the cytoplasmic membrane. Together with roughly 20 other proteins it carries out the cytokinetic process. New cytoplasmic proteinaceous filaments have been detected employing cryo-electron tomography.

For instance, cryo-electron tomography of *C. crescentus* and of the spiral-shaped wall-less bacterium *S. melliferum* has demonstrated the presence of presumed proteinaceous filaments that may traverse the length of the cell. Though their chemical identity in *C. crescentus* (**Figure 25**) has not yet been elucidated, they persist in MreB and crescentin deletion mutants. Crescentin is a *C. crescentus* protein, resembling proteins of eukaryotic intermediate filaments, which is located at the concave side of the cell. Presumably, it functions in maintaining the curved cell shape because cells become straight in its absence.

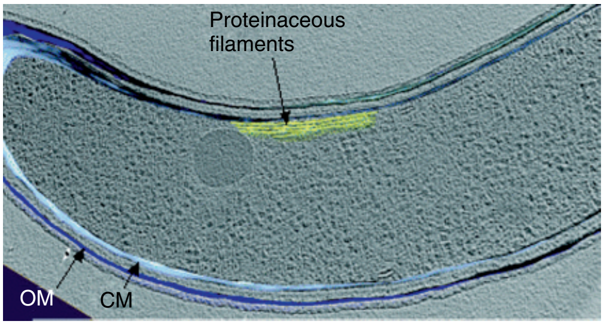

Figure 25 Three dimensionally segmented view of *Caulobacter crescentus* as based on cryo-electron tomography. Color has been added. Proteinaceous filaments have been indicated in yellow. CM, cytoplasmic membrane; OM, outer membrane. Reproduced from Briegel A, Dias DP, Jensen RB, Frangakis AS, and Jensen GJ (2006) Multiple large filament bundles observed in *Caulobacter crescentus* by electron-cryo tomography. *Molecular Microbiology* 62: 5–14.

In *S. melliferum*, cryo-electron tomography has shown three filament bundles traversing the length of the helical cell. Two of them have been supposed to be composed of MreB. The chemical nature of the third filament is not yet known. Three-dimensional reconstructions indicate that movement and change of handedness of the helical organism is accomplished by differential alteration of the MreB filaments (**Figure 26**). So, it seems that the MreB endoskeleton because of its disposition compensates for the absence of a cell wall. The above examples demonstrate

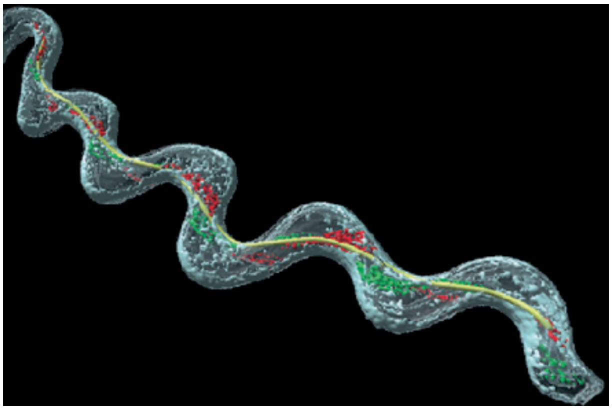

Figure 26 Three-dimensional visualization of *Spiroplasma melliferum.* In yellow is shown the geodetic line, that is, the shortest line between two points (at the cell extremities) within the outline of the cell. Two proteinaceous ribbons are indicated in green and red. The red structures comply more with the geodetic line, indicating that they are shorter than the green ones. This difference is thought to contribute to helicity of the cell. Reproduced from Kürner J, Frangakis AS, and Baumeister W (2005) Cryo-electron tomography reveals the cytoskeletal structure of the Gram-negative bacterium *Spiroplasma melliferum*. *Science* 307: 436–438.

how the application of cryo-electron tomography has revealed new polymeric cytoplasmic structures.

Another example of proteinaceous cytoplasmic filaments is the DNA segregation apparatus of the larger (ChrI) of the two *V. cholerae* chromosomes. Duplicated ChrIs are moved apart by polymerized ParAI proteins. This structure should be considered cytoplasmic because it is not part of the nucleoid.

The Nucleoid

Overall Structure of the Nucleoid (Nucleoplasm)

The nucleoplasm is the central area in the cell that contains the genetic material. Its outline is often irregular in the electron microscope (**Figure 27**), which is presumably determined, at least in part, by the presence of transcripts at the interface of nucleoplasm and cytoplasm. Proteins that play a role in DNA compaction, DNA replication, and transcription are most likely to be located at the periphery of the nucleoplasm, because they are excluded from the DNA-rich region (see below). Whereas, the term nucleoplasm denotes a microscopically visible area in the cell, the nucleoid or bacterial chromosome represents a separate entity that can be isolated or analyzed genetically.

Microscopic and biophysical studies on the nucleoid have been most extensively carried out with *E. coli*. The information, which follows, is therefore largely based on data from this organism. A main problem to solve for a bacterial cell is to compact its DNA while DNA replication, DNA segregation, and transcription can still proceed coordinately. The compaction problem can be illustrated by realizing that the circumference of the circular *E. coli* chromosome is 1.6 mm (4.6 million base pairs), whereas the diameter of the cell is in the order of 1 μm. Assumedly, packing is achieved by a combination of three mechanisms (**Figure 28**). First, the bacterial chromosome is negatively supercoiled, which has been shown most clearly in electron micrographs of spread DNA (**Figure 29**). A supercoiled region is shown schematically

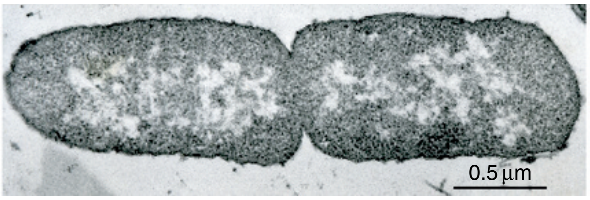

Figure 27 Electron micrograph of a thin section of *Escherichia coli*. This image serves to illustrate the irregular outline of the electron-transparent nucleoplasmic region and the close contact of the latter with the cytoplasm. Courtesy of Dr. CL Woldringh.

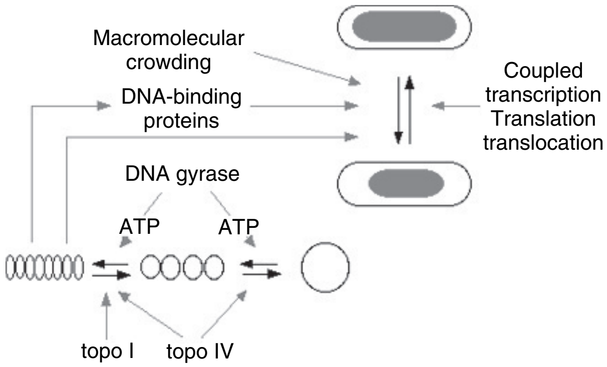

Figure 28 Factors involved in compaction and loosening of the nucleoid. Compaction is thought to occur through macromolecular crowding, DNA-binding proteins, and proteins that affect superhelicity. Loosening might occur through coupled transcription, translation, and protein translocation (cf. **Figure 1**). Reproduced from Stuger R, Woldringh CL, van der Weijden CC, *et al*. (2002) DNA supercoiling by gyrase is linked to nucleoid compaction. *Molecular Biology Reports* 29: 79–82.

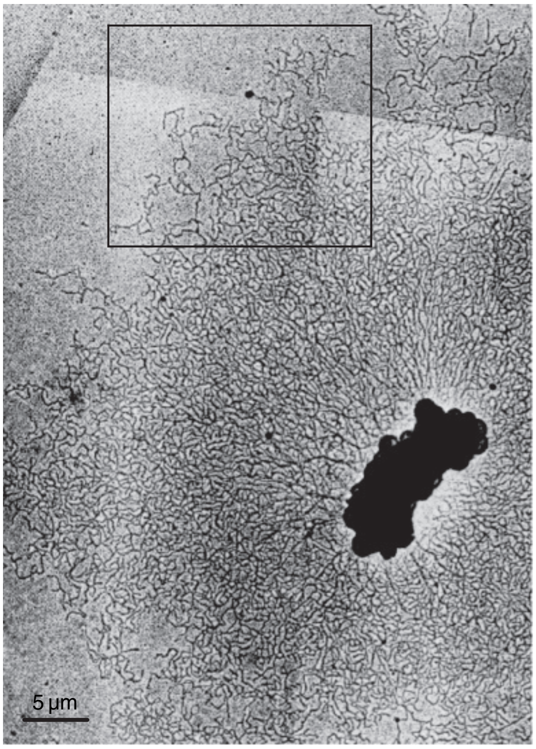

Figure 29 Electron micrograph of an envelope-bound *Escherichia coli* nucleoid spread according to the cytochrome c monolayer technique (Kleinschmidt technique). In the rectangular area, supercoiling is visible at the periphery of the spread nucleoid. Reproduced from Meyer M, de Jong MA, Woldringh CL, and Nanninga N (1976) Factors affecting the release of folded chromosomes from *Escherichia coli*. *European Journal of Biochemistry* 63: 469–475.

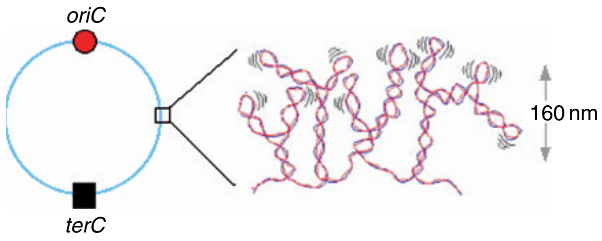

Figure 30 Detail of a supercoiled chromosomal stretch of DNA. The supercoil segments have a persistence length of 160 nm as indicated. The local flexibility of supercoils has been depicted by the short repetitive lines. Reproduced from Woldringh CL and Nanninga N (2006) Structural and physical aspects of bacterial chromosome segregation. *Journal of Structural Biology* 156: 273–283.

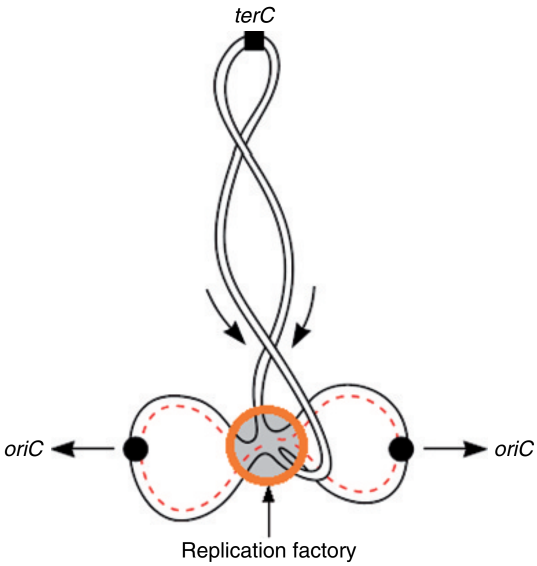

Figure 31 Factory model of DNA replication. DNA to be replicated is threaded through a stationary replication factory. The origins of replication (*oriC*) move apart from each other through an unknown segregation mechanism. After finishing replication, the terminus (*terC*) ends up at one pole, the origin at the other. Consequently, gene regions are not randomly located in the cell. Reproduced from Dingman CW (1974) Bidirectional chromosome replication: Some topological considerations. *Journal of Theoretical Biology* 43: 187–195.

in **Figure 30**. Supercoiling is produced by DNA gyrase and it divides the chromosome in roughly 100 domains. Second, DNA-binding proteins such as the histone-like protein HU, integration host factor IHF, factor for inversion stimulation Fis, and the nucleoid-associated protein H-NS are likely to further reduce the chromosomes' spatial dimensions. Whereas HU can bend and compact DNA at nonspecific sites, IHF can also bind specifically as does Fis. H-NS accomplishes compaction by bridging DNA regions. Thirdly, it is thought that a physical process as phase separation due to macromolecular crowding creates an interface between cytoplasm and nucleoplasm. The relative contributions of these three compaction mechanisms to chromosome folding are still a matter of debate. Clearly, they should not create a static structure and they should allow for a dynamic positioning of bacterial genes (see below). Note that bacteria do not posses nucleosomes or nucleosome-like structures. By contrast, archaea have nucleosomes that are similar to their eukaryotic counterparts. This underlines another evolutionary distinction between bacteria and archaea.

Substructure of the Nucleoid

In recent years, considerable advances have been made regarding the cellular positioning of the DNA replication machinery and specific gene regions, such as origin of replication (*oriC*) and terminus (*terC*; **Figure 30**). This is largely due to the application of fluorescence microscopy, be it with immunolabeling or by visualizing green fluorescent protein (GFP) fusions in living cells. Most cytological advances have been made with slow growing cells, which exclude the complication of multifork replication. Multifork replication arises when the doubling time of the culture is smaller than the duration of DNA replication. By labeling components of the DNA replication machinery, it has been deduced that DNA replication is effected in a central cellular compartment. In this compartment, the two replication forks of the bidirectional replicating chromosome are in the vicinity of each other. In other words and in accordance with the earlier proposals, the DNA to be replicated is threaded through a stationary replication factory (**Figure 31**). Supporting this model is the observation that duplicated *oriC*s move in opposite direction toward the poles. Simultaneous labeling of *oriC* and *terC* has revealed that they occupy distinct cellular positions dependent on the progress of DNA replication during the cell cycle. Labeling of gene regions on intermediate positions on one arm of the circular chromosome has shown that the label also occurs at intermediate positions. An arm is here defined as the chromosomal segment between *oriC* and *terC* (cf. **Figure 30**). In a more detailed analysis comparing the cellular localization of genes on the arbitrary left and right arm of the chromosome, it has been found that the left arm and the right arm show a defined arrangement with respect to the length axis of the cell (**Figure 32**). These results make it clear that the gene position in the nucleoid is not at all random. Their position is dynamic in the sense that it depends on the DNA replication stage of the bacterial chromosome (cf. **Figure 31**).

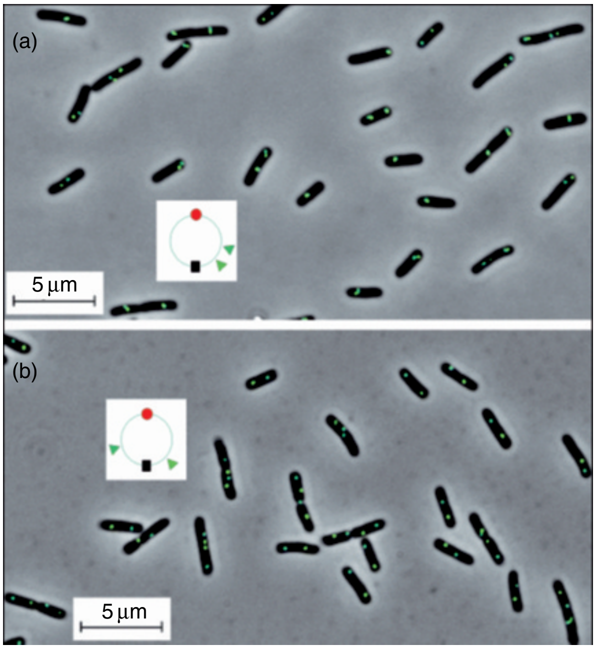

Figure 32 Dual fluorescent label of chromosomal regions in *Escherichia coli.* (a) Two chromosomal sites have been labeled on the arbitrary right arm (between origin and terminus, cf. **Figure 30**). (b) Labeling of chromosomal sites on the left and right arms. Reproduced from Nielsen HJ, Li Y, Youngren B, Hansen FG, and Austin S (2006) Progressive segregation of the *Escherichia coli*. chromosome. *Molecular Microbiology* 62: 331–338.

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Chromosome, Bacterial

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Defining Statement
Historical Introduction
Chromosome Form and Number
Gene Arrangements
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DNA Twisting, Folding, and Bending
Chromosome Inactivation
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Glossary

DNA supercoiling A phenomenon occurring in constrained duplex DNA molecules when the number of helical turns differs from the number found in DNA molecules of the same length, but containing an unconstrained end that can rotate. Supercoiling creates strain in constrained DNA molecules. A deficiency of duplex turns generates negative supercoiling; a surplus generates positive supercoiling. Supercoils can be helical or plectonemic (similar to a twisted rubber band).

DNA topoisomerases Enzymes that change DNA topology by breaking and rejoining DNA strands. Topoisomerases introduce and remove supercoils, tie and untie knots, and catenate and decatenate circular DNA molecules.

genome The entire complement of genetic material in a bacterium or in the nucleus, mitochondrion, or chloroplast of a eukaryotic species.

nucleoid A term for the bacterial chromosome when it is in a compact configuration, either inside a cell or as an isolated structure.

origin of replication A location on the chromosome (*oriC*) where initiation of replication occurs. For *E. coli*, *oriC* is about 250 nt long and during initiation it specifically interacts with several proteins to form an initiation complex. Archaebacterial chromosomes may contain as many as three replication origins.

recombination A process in which two DNA molecules are broken and rejoined in such a way that portions of the two molecules are exchanged.

replication fork The point at which duplex DNA separates into two single strands during the process of DNA replication. Associated with replication forks are DNA helicases to separate the strands and DNA polymerases to synthesize new DNA strands.

Abbreviations

FIS	factor for inversion stimulation
H-NS	histone-like nucleoid structuring protein
IHF	integration host factor
LRP	leucine-responsive regulatory protein

Defining Statement

Advances in microscopy reveal intracellular locations and movements of specific chromosome regions. DNA conformations (supercoiling, folding, and looping) are also dynamic. Nucleotide sequence analysis reveals many historical additions, deletions, and rearrangements; strains within a species can display both common and diverse sequences.

Historical Introduction

Bacterial chromosomes were discovered much later than their eukaryotic counterparts, largely due to their small size. Moreover, bacterial chromosomes do not undergo the striking metaphase condensation that makes eukaryotic chromosomes so easy to see. Indeed, it was not until the early 1940s that bacteria were clearly shown to undergo spontaneous mutation and to have mutable genes. At about that time, Avery and associates discovered the chemical nature of genetic material: extracted DNA carried a character for polysaccharide synthesis from one strain of *Pneumococcus* to another. At first, the result was not universally accepted as evidence for genetic exchange, partly because the so-called 'transforming principle' exerted its effect after an unknown number of steps and partly because Avery lacked a molecular framework for explaining how DNA could function as genetic material. In 1952, Hershey and Chase announced that phage DNA, not protein, is injected into bacterial cells during infection, and a year later Watson and Crick provided the structural framework for DNA. At that point DNA became widely accepted as the carrier of hereditary information, and a search for bacterial chromosomes began.

By 1956, nucleoids, as bacterial chromosomes are called, could be seen in living cells as discrete, compact structures (for recent example see **Figure 1**). Gentle extraction methods eventually yielded large, intact DNA molecules; by the early 1970s it became possible to isolate a compact form of the chromosome for biochemical study. DNA supercoiling had been discovered in the mid-1960s, and within a decade enzymes called DNA topoisomerases that introduce and remove supercoils were found. The existence of DNA topoisomerases gave credence to the idea that chromosomal DNA is under torsional tension inside cells. During the 1980s the dynamic, regulated nature of supercoiling emerged as a major structural feature that needed to be considered whenever the activities of the chromosome were discussed. The development of rapid methods for determining nucleotide sequences led to complete sequences for many bacterial genomes in the 1990s. DNA sequence analyses led to the conclusion that all living organisms share a common ancestor, and inferences could be drawn about the nucleotide sequence history of chromosomes.

An emerging theme is the dynamic nature of bacterial chromosomes. In terms of nucleotide sequence, massive gene shuffling has occurred over the course of evolution. With respect to three-dimensional structure, portions of the chromosome move to particular regions of the cell at specific times during the cell cycle, while the bulk of the DNA threads through replication forks. At the level of DNA conformation, changes can occur within minutes after alterations in cellular environment occur. These changes are influenced, and in some cases directed, by protein components of chromosomes.

In the following sections we sketch major concepts concerning chromosome structure. We emphasize that a bacterial chromosome is not equivalent to a bacterial genome: a chromosome is a dynamic protein–RNA–DNA structure that can vary in conformation, size, DNA content, and form with growth conditions, whereas a genome is the genetic information content of the organism, its DNA sequence; a genome does not change with growth conditions.

Chromosome Form and Number

Bacterial DNA has been found in both circular and linear forms. For *Escherichia coli*, chromosomal circularity is supported by three lines of evidence. First, circles were observed when radioactively labeled DNA was extracted from cells and then examined by autoradiography. Although almost all of the molecules in these experiments

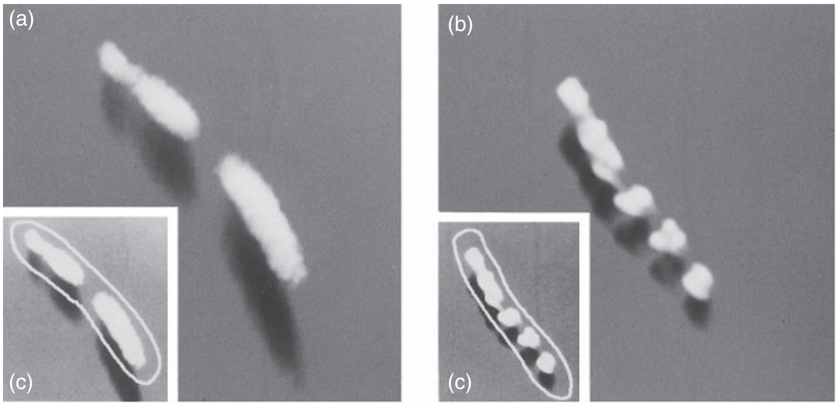

Figure 1 Bacterial nucleoids. Nucleoids of *Escherichia coli* K-12 were visualized in a confocal scanning laser microscope as developed by GJ Brankenhoff (*Nature* 317: 748–749, 1985). Elongated cells were obtained by growth in broth. Then the nucleoids were stained with the DNA-specific fluorochrome DAPI (0.1 μg ml^{-1}) added to the growth medium. Under these conditions the stain had no effect on growth. The cells were observed either alive (a) or after fixation with 0.1% osmium tetroxide (b). Since the cell boundary is not easily visualized, it has been sketched in for reference (c). Multiple nucleoids were present because these fast-growing cells contain DNA in a state of multifork replication. In live cells the nucleoid has a cloud-like appearance and a smooth boundary with the cytoplasm (protuberances, if present, would be smaller than 200 nm). Magnification for panels (a) and (b) is 9000×. Photo courtesy of Dr. Conrad Woldringh, Department of Molecular Cell Biology, University of Amsterdam, The Netherlands.

were so tangled that their configurations were unclear, a few appeared as large circles more than a millimeter in length (cells are only 1 or 2 μm long). However, these circular molecules exhibited a size range of severalfold, which was not readily explained. Second, genetic mapping studies are most easily interpreted as the genes being arranged in a circle, although a linear interpretation is still possible (mapping can be ambiguous, since a large linear bacteriophage DNA is known to have a circular genetic map). Third, two bidirectional replication forks emerge from a single origin of replication, and DNA moves through a 'replisome' (or the replisome moves through DNA) such that the forks converge at a point located 180° opposite to the origin on the circular map. Recombination and decatenation events expected to be associated with large circular DNA then allow each daughter cell to inherit a chromosome. Conclusive evidence for circularity would be visualization of circular images for most of the DNA molecules present.

In 1989, chromosomal DNA molecules of *Borrelia burgdorferi* were found to have a linear form. Linear chromosomes were subsequently observed in *Streptomyces* species, *Rhodococcus fascians*, and *Agrobacterium tumefaciens*. With *Streptomyces*, DNA ends contain repetitive sequences as well as terminal proteins that prime DNA synthesis complementary to the 3′ end of the DNA. In *B. burgdorferi*, the ends are hairpins that facilitate complete replication. Thus bacteria have chromosomal ends that function much like the telomeres of linear chromosomes in eukaryotic cells.

Many bacteria carry all of their genes in a single genetic linkage group, as if they have a single type of chromosome. However, there is a growing list of species in which useful or essential genes are found on two or more chromosomes. The number of large, circular-mapping DNA molecules is two for *Vibrio cholerae*, *Leptospira interrogans*, *Rhodobacter sphaeroides*, and *Brucella* species; three for *Rhizobium meliloti*; and 2–4 among the isolates of *Burkholderia* (*Pseudomonas*) *capecia*. Some *Agrobacterium* species contain one circular- and one linear-mapping chromosome. Thus the old idea that prokaryotes contain only one circular chromosome has been abandoned. Indeed, those with more than one chromosome (genetic linkage group) may constitute a sizable class, since the vast majority of bacterial species have yet to be examined.

Distinguishing between chromosomes and plasmids can be difficult, since some plasmids are very large and contain genes essential for cell growth. Moreover, some large plasmids integrate into, and excise from, chromosomes. Thus chromosome number in some species may be variable.

The existence of multiple copies of chromosomal regions, as well as entire chromosomes (multiploidy), is well known in eukaryotes. In addition, a eukaryotic cell can contain thousands of copies of mitochondrial and chloroplast genomes. Multicopy genomes are also common among bacteria. For example, the cells of *E. coli* growing rapidly in a rich medium (20-min doubling time) at low cell density are large and contain about ten genome equivalents of DNA per cell, whereas the number is between one and two in small cells during slow growth. Cells of *Deinococcus radiodurans*, contain ten genome equivalents during exponential growth and four during the stationary phase. Even slowly growing cells, such as *Borrelia hermsii* (minimum doubling time 8 h), can carry multiple genomes. This bacterium contains 8–11 genome copies when grown *in vitro* and up to 16 copies when grown in mice. *Azotobacter vinlandii* presents a dramatic example. Genome copy number in rich medium increases from 4 to 40 and then to greater than 100 as the culture progresses from early exponential through late exponential to stationary phase. DNA per cell then decreases at the start of a new growth cycle. The spectacular increase in genome copy number in *Azotobacter* is not observed with cells grown in minimal medium. High copy number may result from an 'engorge now divide later' reproductive strategy. When nutrients are abundant, it might be advantageous to carry many genomes in large cells to be diluted into smaller cells when nutrients become limited. An extreme practitioner of this strategy is the eubacterium *Epulopiscium fishelsoni*, which can fill its half-millimeter-long cells with 100 000 genome-equivalents of DNA in times of plenty. When nutrients run out, the large cells subdivide into many smaller cells.

Regardless of the reason for genome multiplicity, it is clearly not restricted to eukaryotic cells. Indeed, bacterial and eukaryotic chromosomes can no longer be considered different with respect to form (both types can be linear) and number of linkage groups (bacteria, which often have one, can have several; eukaryotes, which usually have many chromosomes, can have only one, as seen with the ant *Myrmecia pilosula*). What distinguishes bacterial and eukaryotic chromosomes is the coupling between replication and segregation: it is flexible in bacteria and tight among eukaryotes As a result, a nuclear chromosome never contains more than one genome equivalent of DNA as it segregates to daughter cells. In contrast, a bacterial chromosome may contain from one to many genome equivalents of DNA (depending on growth conditions) even during segregation.

Gene Arrangements

Gene mapping in bacteria was originally based on the ability of an externally derived, genetically marked fragment of DNA to recombine with the homologous region of a recipient's DNA. The frequency with which two nearby markers recombine is roughly proportional to the distance between them. As mutations were collected for a variety of purposes, characterization of a mutation usually included determining its map position on the genome. The resulting genetic maps revealed relationships among genes such as operon clusters, showed orientation preferences that might reflect chromosomal activities, and suggested that some chromosomal information may have been derived from plasmids and phage. The discovery of restriction endonucleases led to a quantum advance in genetic mapping, since these enzymes allowed the accurate construction of maps in terms of nucleotide distances. Practical nucleotide sequencing methods, which became available in the late 1970s, were expected to yield the complete nucleotide sequences for at least 1000 bacterial species by the year 2008. Data are being obtained at three levels: (1) the genetic map, with the genes and their map locations correlated with the role of the gene products in cell metabolism, structure, or regulation, (2) the physical map in terms of locations of restriction sites, and (3) the nucleotide and corresponding protein amino acid sequences. It is becoming clear that all living organisms probably arose from a common ancestor. Thus information on nucleotide sequence and gene function in one organism can be applied to many other organisms.

The conservation of gene structure makes it possible to use nucleotide sequence information for comparison of genetic maps among bacterial species. One of the features revealed is clustering of related genes. For example, the genetic maps of *Bacillus subtilis*, *E. coli*, and *Salmonella typhimurium* show a grouping of many genes for biosynthetic and degradative pathways. Such grouping could be for purposes of coordinated regulation, since some adjacent genes produce polycistronic messages. A completely different view of the same data maintains that functionally related genes move horizontally (from one organism to another) as clusters, because the products of the genes work well together, increasing the probability of successful transfer. Both ideas are likely to be accurate.

While it is clear that genomes are quite malleable, the time frame over which insertions and deletions occur can be large. Some perspective is provided by comparison of the maps of *E. coli* and *S. typhimurium.* One large genetic inversion occurred, and the maps have major differences at about 15 loci where pieces of DNA were either inserted into or deleted from one genome or the other. But the overall arrangement of gene order between the organisms is remarkably conserved. In contrast, the genomes of some other bacteria, such as *Salmonella typhi*, *Helicobacter pylori*, and strains of *Pseudomonas aeruginosa*, *Fransciella tularensis*, and *Bartonella henselae*, appear to have undergone substantial rearrangement. Interestingly, when *S. typhi* and *S. typhimurium* are grown in the laboratory, rearrangements are found for both, but when isolated from humans from all over the world, rearranged genomes are found for *S. typhi* but not *S. typhimurium.* The reason for differences among organisms is not clear. Some rearrangements, such as insertions or deletions, may be more deleterious for certain bacteria. Alternatively, the opportunity for rearrangements, such as the occurrence of recombinational hot spots, may be greater in some organisms than in others. The latter explanation appears to be more likely for chloroplast and mitochondrial genomes, which in many ways resemble bacterial genomes. After some 300 million years of evolution, the order of chloroplast genes is highly conserved among most land plants, including mung bean. Yet chloroplast gene order is completely scrambled in pea, a plant closely related to beans. Massive rearrangement of genes is also evident when mitochondrial genome maps are compared among types of maize. Thus gene order in organelles appears to have little functional significance, and it can be subject to frequent recombination if the opportunity arises. Situations in which the opportunity is lacking clearly exist.

As complete genomic nucleotide sequences become increasingly available, new questions arise. For example, what is the minimal number of genes required for independent life? Endosymbionts of sap-sucking insects currently hold the record: *Carsonella ruddii* DNA has only 160 000 bp arranged in 182 open reading frames. Nucleotide sequence analysis is also identifying genes involved in pathogenicity by comparison of virulent and avirulent strains of a pathogen. For example, such an approach has uncovered a ‘pathogenicity island’, a collection of virulence genes, in *H. pylori.* The island is bounded by 31 bp direct repeats, as if it had been transferred horizontally into an ancestor of *H. pylori.* In some bacteria, horizontal transfer may have been quite extensive. With *E. coli* as much as 15% of the genome, 700 kbp, may have been acquired from foreign sources such as integrative bacteriophage, transposons in plasmids, and conjugative transposons (genetic elements that cannot replicate independently but cause the occurrence of conjugation, a form of cell-to-cell DNA transfer).

Recombination

Intracellular DNA experiences a variety of perturbations that must be repaired to maintain the integrity of the chromosome and to allow movement of replication forks. Cells have several ways to repair DNA damage, one of which involves recombination (Recombination is a process in which DNA molecules are broken and rejoined in such a way that portions of the two molecules are exchanged.) Damaged sequences in one molecule can be exchanged for undamaged ones in another. It is now thought that the *raison d’être* for recombination is its role in DNA repair, a process that occurs thousands of times per cell generation.

Recombination is also involved in DNA rearrangements arising from the pairing of repeated sequences. When the repeats are in direct orientation, duplications and deletions arise; inversions arise when the repeats are inverted. In *B. subtilis* a cascade of sequential rearrangements has been identified in which large transpositions and inversions have been attributed to recombination at specific junction points in the genome. Several other examples are found for the *rrn* clusters, sets of similar assemblies of ribosomal and transfer RNA genes. Rearrangements at the three *rrn* clusters of *Brucella* are thought to be responsible for differences in chromosome size and number among species of this genus. Other repeated sequences that facilitate rearrangements are duplicate insertion sequences, the *rhs* loci (*r*ecombination *h*ot *s*pot), and experimentally introduced copies of the transposon Tn10.

The third consequence of recombination is the insertion of genes from mobile elements into chromosomes. These elements, which include transposons, plasmids, bacteriophage, integrons, and pathogenicity islands, move from one cell to another and sometimes from one species to another. A spectacular example of gene transfer and its evolutionary effect across kingdoms is seen in the acquisition of a 500 kbp symbiosis island. This island of DNA converts a saprophytic *Mesorhizobium* into a symbiont of lotus plants that is capable of fixing nitrogen. Because they mediate such sweeping change, mobile genetic elements may represent the most important means for generating the genetic diversity on which selection operates. For mobility, and thus generation of genetic diversity, such genetic elements require recombination activities.

DNA Twisting, Folding, and Bending

DNA Supercoiling

Circular DNA molecules extracted from mesophilic bacteria have a deficiency of duplex turns relative to linear DNAs of the same length. This deficiency exerts strain on DNA, causing it to coil. The coiling is called negative supercoiling (an excess of duplex turns would give rise to positive supercoiling). Negative supercoils can assume either a helical or a plectonemic form (the latter is similar to a twisted rubber band). Superhelical strain is spontaneously relieved (relaxed) by nicks or breaks in the DNA that allow strand rotation; consequently, supercoiling is found only in DNA molecules that are circular or otherwise constrained so the strands cannot rotate. Since processes that separate DNA strands relieve negative superhelical strain, they tend to occur more readily in supercoiled than in relaxed DNA. Among these activities are the initiation of DNA replication and initiation of transcription. Negative supercoiling also makes DNA more flexible, facilitating DNA looping, wrapping of DNA around proteins, and the formation of cruciforms, left-handed Z-DNA, and other non-B-form structures. In a sense, negatively supercoiled DNA is energetically activated for most of the processes carried out by the chromosome.

Negative supercoils are introduced into DNA by gyrase, one of the several DNA topoisomerases found in bacteria. DNA topoisomerases act through a DNA strand breaking and rejoining process that allows supercoils to be introduced or removed, DNA knots to be tied or untied, and separate circles of DNA to be linked or unlinked. The action of gyrase is countered by the relaxing activities of topoisomerase I and topoisomerase IV. Since gyrase is more active on a relaxed DNA substrate, while topoisomerases I and IV are more active on a negatively supercoiled one, the topoisomerases tend to reduce variation in supercoiling. Moreover, lowering negative supercoiling raises gyrase expression and lowers topoisomerase I expression. Thus negative supercoiling is a controlled feature of the chromosome.

Supercoiling is influenced by the extracellular environment. For example, when bacteria such as *E. coli* are suddenly exposed to high temperature, negative supercoiling quickly drops (relaxes), and within a few minutes it recovers. The reciprocal response is observed during cold shock. Presumably these transient changes in DNA supercoiling facilitate timely induction of heat- and cold-shock genes important for survival. Supercoiling is also affected by the environment through changes in cellular energetics. Gyrase hydrolyses ATP to ADP as a part of the supercoiling reaction, and ADP interferes with the supercoiling activity of gyrase while allowing a competing relaxing reaction to occur. Consequently, the ratio of [ATP] to [ADP] influences the level of supercoiling. Changes in oxygen tension and salt concentration provide examples in which cellular energetics and supercoiling change coordinately. Collectively, these observations indicate that chromosome structure changes globally in response to the environment.

Supercoiling is influenced locally by transcription. During movement of transcription complexes relative to DNA, RNA polymerase does not readily rotate around DNA. Consequently, transcription generates positive supercoils ahead of the polymerase and negative supercoils behind it. Since topoisomerase I removes negative supercoils and gyrase positive ones, transcription and similar translocation processes have only transient effects on supercoiling. However, cases in which induction of very high levels of transcription results in abnormally high levels of negative supercoiling have been found. In such situations transcription-mediated changes in supercoiling provide a way for specific regions of a DNA molecule to have levels of supercoiling that differ greatly from average values.

Problems can arise when negative supercoils build up behind a transcription complex and facilitate DNA strand separation, since nascent transcripts form long hybrids with the coding strand of DNA. Such hybrid structures, called R-loops, can interfere with gene expression. These hybrids are removed by ribonuclease H; consequently, a deficiency of topoisomerase I can be corrected by overexpression of ribonuclease H. Problems also arise from a buildup of positive supercoils ahead of a transcription complex, since helix tightening will slow transcription. That is probably why strong gyrase-binding sites are scattered throughout the chromosome immediately downstream from active genes.

DNA Looping

In the early 1970s Worcel showed that multiple nicks are required to relax chromosomal supercoils, demonstrating that DNA must be constrained into topologically independent domains. Superhelical tension and topological domains were later detected in living cells, making it unlikely that the domains are artifacts of chromosome isolation. Dividing supercoiled DNA into independent domains keeps a few nicks or breaks from relaxing all the supercoils. Independent domains also allow supercoils to be introduced into the chromosome before a round of replication finishes – in the absence of domains, the gaps following replication forks would relax any supercoils that gyrase might introduce. While early studies estimated the number of domains at about 50–100, more recent work, some based on site-specific DNA breakage and supercoil-sensitive expression of particular genes, places the number in the hundreds, about one per 10 kb of DNA.

A variety of processes probably contribute to domain structure. One may be coupled transcription–translation of proteins that transiently anchor the chromosome to the cell membrane. This process, called transertion, restricts strand rotation. Since domain barriers are present even when RNA synthesis is inhibited, additional factors, such as the MukBEK protein, are likely to be involved. MukBEK has two DNA-binding domains connected by a long flexible linker. Thus, it could restrict DNA rotation by binding to distant regions of DNA. Another type of constraint is seen after exhaustive deproteinization. Nearly every nucleoid in preparations from both exponential and stationary-phase *E. coli* appears by fluorescence microscopy as a rosette or loose network of 20–50 large loops. Such interactions involving only DNA may reflect the role of recombination in forming some of the domains. Still other factors may be the DNA-compacting proteins discussed below.

DNA-Compacting Proteins

Five small, abundant, DNA-binding proteins have captured attention as possible elements of chromosome packaging. At one time these proteins were called histone-like proteins, but they have little resemblance to histones with respect to amino acid sequence. They have also been called nucleoid-associated proteins, but that term encompasses many more proteins. A unifying feature is the ability of these proteins to compact DNA. A variety of functions, including regulation of gene expression and in several cases participation in site-specific recombination, have evolved for these proteins. Most of our knowledge about the proteins comes from biochemical studies.

The most abundant of the small compacting proteins is HU (60 000–100 000 monomers per cell). Each member of the dimer protein has a long arm; together the arms reach around DNA, using a proline at the tip of each arm to intercalate into DNA and either create or stabilize kinks in DNA. HU binding, which lacks nucleotide sequence specificity, shows a preference for supercoiled DNA. HU constrains negative supercoils, which led to the idea that the protein wraps DNA into nucleosome-like particles *in vitro*. Nucleosomes, which have long been recognized as a distinctive feature of eukaryotic nuclei, are ball-like structures in which about 200 bp of DNA is wrapped around histone proteins. Nucleosomes occur at regular intervals along DNA, giving nuclear chromatin a "beads-on-a-string" appearance. True bacteria (eubacteria) do not have true histones or nucleosomes, although some archaebacteria do. Thus HU is more likely to be a bending rather than a wrapping protein. With some DNAs, HU introduces a 180° bend, although on average the bends are closer to 100°. The bending activity is especially clear when HU serves as an architectural protein, assisting in the formation of DNA–protein complexes that carry out site-specific recombination. HU also provides the DNA bending needed for certain repressors to bring distant regions of DNA together in loops that block initiation of transcription.

Closely related to HU is a bending protein called IHF (integration host factor, 30 000–60 000 copies per cell). It bends DNA roughly 160°, but unlike HU, IHF recognizes specific nucleotide sequences (about 1000 specific IHF-binding sites are present in the *E. coli* genome). Many examples have been found in which IHF helps form a DNA loop between promoters and transcription activators located far upstream from promoters. IHF also participates as an architectural protein during the formation of site-specific DNA–protein complexes. The best known of these is the intasome generated by bacteriophage lambda during integration into and excision from the bacterial chromosome. Since cells contain many more copies of IHF than specific IHF-binding sites, the protein may also have a nucleotide-sequence-independent mode of binding that contributes to DNA compaction. *In vitro* the protein can compact DNA by 30%, presumably through nonspecific binding.

The third protein is called FIS (factor for inversion stimulation). FIS recognizes a weakly conserved 15 bp binding site that is present at almost 6000 copies per genome. FIS is a dimer of two identical subunits that appear to bind in adjacent major grooves of DNA. A bending angle of about 50° to 90° is generated as dimerization of the protein pulls on the two portions anchored to DNA. FIS also binds nonspecifically to plectonemic supercoiled DNA, clustering at DNA crossover points and at the apexes of DNA loops. Thus, FIS may stabilize DNA loops.

The level of FIS expression is sharply elevated shortly after dilute bacterial cultures enter logarithmic growth, reaching a maximal copy number of about 60 000 per genome. In older stationary-phase cultures, the rate of FIS synthesis drops to almost zero. Some of the binding sites for FIS are so close to promoters that FIS acts as a repressor. In other cases, FIS acts as an upstream activator of transcription. It also serves as an architectural protein when it forms protein–DNA complexes for site-specific recombination.

LRP (leucine-responsive regulatory protein; 3000 dimers per cell) is a small protein that responds to the nutrient status of the cell, particularly amino acid levels. It acts as a repressor for many genes involved in catabolic (breakdown) processes and as an activator for genes involved in metabolic synthesis. LRP appears to have two modes of action. At its own promoter it oligomerizes into an octomeric, nucleosome-like structure that wraps DNA. The second mode is much like the DNA bridging observed with H-NS (see below). As with HU, IHF, and FIS, LRP has architectural roles when it forms protein–DNA complexes for site-specific recombination.

The fifth protein is called H-NS (histone-like nucleoid structuring protein, 20 000 molecules per cell). Unlike the four other small DNA-compacting proteins, H-NS does not actively bend or wrap DNA. Instead, it binds to DNA that is already bent, generally at AT-rich sequences; thus, H-NS stabilizes DNA bends. H-NS is composed of two domains connected by a flexible linker. The N-terminal domain dimerizes with another H-NS molecule, while the C-terminus binds DNA. Thus the dimer has two DNA-binding domains. Electron microscopy studies indicate that H-NS can form bridges between regions of double-stranded DNA, while biochemical work suggests that binding of H-NS stiffens DNA, perhaps in local patches. H-NS patches interacting with each other could be responsible for the DNA bridging effect, which could stabilize DNA loops. If an appropriate DNA bend is near the promoter of a gene, H-NS binding will repress the gene by preventing RNA polymerase binding. Binding within a gene can also act as a roadblock to transcription, and with some genes the bridging function of H-NS appears to trap RNA polymerase in a DNA loop. The expression of hundreds of genes is likely to be affected by H-NS. An interesting possibility is that H-NS binds to many regions of DNA when they enter a genome ‘horizontally’, thereby repressing large numbers of genes. Such gene silencing would allow cells to accept a new piece of DNA that might otherwise express deleterious genes.

While the abundance and biochemical properties of DNA-compacting proteins make them good candidates for chromosome structural elements, the dynamic nature of their interactions with DNA and their multiple, sometimes redundant activities make it difficult to assign firm roles in DNA packaging. Nevertheless, combinations of compacting protein mutations are associated with reduced nucleoid compaction, as seen with HU/FIS double mutants. Conversely, overexpression of H-NS increases compaction.

Chromosome Inactivation

In eukaryotic cells, large portions of genomes are rendered transcriptionally inactive by heterochromatinization, a local DNA compaction that is readily observed by light microscopy. Bacterial chromosomes are too small to see locally compacted regions; consequently, we can only guess about their existence. However, evidence is accumulating that bacteria have systems that condense entire chromosomes. *Caulobacter crescentus* serves as an example. In the life cycle of this organism, two cell types exist: swarmer cells and stalk cells. The latter are genetically active. When a swarmer cell differentiates into the stalked type, the nucleoid changes from a compact form into a more open structure, possibly reflecting transcriptional activation of the chromosome. In the second example, a histone H1-like protein in *Chlamydia trachomatis* appears to cause chromosomal condensation when the metabolically active reticulate body differentiates into an inactive, extracellular elementary body. Another example occurs during sporulation in *Bacillus*. In this case the chromosome of the spore is bound with new proteins as its transcriptional activity ceases. Still another case is seen when the archaebacterium *Halobacterium salinarium* progresses from early to late exponential phase of growth. The *H. salinarium* nucleoid, when obtained by gentle lysis, changes from a type containing naked DNA to one having the beads-on-a-string appearance typical of nucleosomal DNA. This change, seen by electron microscopy, is also reflected in nucleoid sedimentation properties. Finally, fluorescence measurements of DNA and RNA within the enormous cells of *E. fishelsoni*, which, as pointed out above, are up to 500 μm long, suggest that decondensation and dispersion of the nucleoid is accompanied by increased transcriptional activity. Whether some of these diverse organisms share a common mechanism for chromosome activation–inactivation is unknown.

Chromosome Duplication and Segregation

The major features of chromosome replication have been established for many years. Semiconservative replication was demonstrated by density-shift experiments in 1958, and a few years later the autoradiograms prepared by Cairns revealed a partially replicated circle containing a large replication 'bubble'. In the early 1970s it became clear that bidirectional replication begins at a fixed origin (*oriC*) with a pair of forks pointing in opposite directions along the genetic map. With *E. coli*, about 40 min is required for the forks to reach a point located 180° from *oriC* on the genetic map, thereby completing the replication of the 4.6 megabase chromosome. The two daughter chromosomes then segregate. Under conditions of rapid growth, bacterial chromosomes can contain more than one pair of replication forks, allowing cells to inherit chromosomes containing more copies of genes near *oriC* than far from *oriC*. The location of highly expressed genes (such as those encoding rRNA and RNA polymerase) near *oriC* may be advantageous for bacteria capable of rapid growth, thereby providing a reason for segregation of branched DNA. In the nucleus of a eukaryotic cell, however, only unbranched DNA molecules that have completed replication can serve as chromosomes (segregating genetic units) during cell division.

Initiation of replication has long been a focus of attention, since it is expected to regulate the cell cycle. Early in the study of initiation, heat-sensitive mutations were obtained in genes called *dnaA* and *dnaC*. These mutations made it possible to uncouple initiation from the elongation phase of replication. Then the origin was cloned by its ability to confer replication proficiency to a plasmid lacking an origin of replication. The availability of *oriC* on a small piece of DNA, plus purified initiation proteins, allowed Kornberg to develop an *in vitro* initiation system. From this system we learned that initiation involves the specific binding of DnaA to *oriC* and the wrapping of origin DNA around the protein. Local DNA strand separation then occurs at the origin, and single-stranded binding protein attaches to the separated strands. That helps stabilize what looks like a single-stranded bubble in duplex DNA. The DnaB helicase, helped by the DnaC protein, binds to the replication bubble and enlarges it. Then DNA polymerase binds to form two replication forks. The two forks point in opposite directions; thus, as DNA synthesis begins, the left half of the chromosome moves through one fork and the right half through the other fork.

Sensitive probes that bind to specific regions of the chromosome are being used to address major cytological questions such as whether DNA is drawn through stationary replication 'factories' or whether the replication machinery moves along the DNA. For a decade the former idea was favoured. Fluorescent labeling of DNA polymerase indicated that replication forks are located at the center of the cell, where they remained throughout most of the cell cycle. When multifork replication occurred, two additional replication centers, each probably containing a pair of forks, were seen situated between the midcell forks and the cell poles. Data from Sherratt recently argued for independent replication forks that follow the path of the DNA in *E. coli* cells. These opposing views have not been resolved.

A second issue concerns the origin of replication (*oriC*), which can be located by fluorescent antibodies directed at proteins that bind to repeated nucleotide sequences placed near *oriC* (or any other specified region). In some studies of newly formed cells, *oriC* and the replication terminus are located at opposite poles of the nucleoid, implying that the dynamic nucleoid must have internal structure. At the beginning of replication, *oriC* moves briefly toward a midcell position, presumably to the replication apparatus. Later, two copies of *oriC* become visible at the same nucleoid pole, apparently having been drawn back to the pole after replication begins. Still later, one copy of *oriC* abruptly moves to the opposite edge of the nucleoid. Eventually the replication terminus is pulled to the replication apparatus at the midcell position, and late in the cell cycle two replication termini can be seen pulling apart. Then the septum that separates new daughter cells forms between the termini. The localization of *oriC* and its rapid movement, which is about 10 times faster than cell elongation, indicate that bacterial chromosomes undergo a form of mitosis. But unlike eukaryotic mitosis, the bacterial chromosome continues to be replicated and transcribed throughout segregation.

While the details of bacterial 'mitosis' are still poorly understood and rapid movement of *oriC* is not always seen, several proteins exhibit properties expected of mitotic proteins. For example, in *B. subtilis* the ParB (SpoOJ) protein appears to participate in chromosome partitioning by binding to multiple sites on the chromosome near *oriC*. An attractive idea is that ParB holds the new and old copies of *oriC* near one pole of the nucleoid until the mitotic apparatus pulls one *oriC* copy to the opposite pole. Since mitosis is expected to be an essential activity, it is surprising that mutations in *parB* are not lethal. Clearly, there is much more to learn about the segregation of sister chromosomes to daughter cells.

We expect DNA tangles to arise as replicated chromosomes pull apart. The double-strand passing activity of gyrase and topoisomerase IV is well suited for resolving tangles, with the movement of daughter chromosomes to opposite cell poles providing the directionality needed by the topoisomerases to untangle loops. Consistent with this idea, both gyrase and topoisomerase IV are distributed around the *E. coli* chromosome, as judged by DNA cleavage induced by the quinolone inhibitors of the topoisomerases. Replication is also expected to leave daughter chromosomes catenated (interlinked). Plasmid studies indicate that unlinking may be a function of topoisomerase IV, although other topoisomerases are also able to perform the function. For example, gyrase shows decatenating activity *in vitro*, as do topoisomerases I and III if nicks or gaps are present in DNA. Some of these backup systems must function in *Mycobacterium tuberculosis*, *Treponema pallidum*, and *H. pylori*, since these bacteria lack topoisomerase IV.

Chromosome Packaging Dynamics

Nucleoid compaction occurs at four levels. One is macromolecular crowding: cytoplasmic proteins and other large cytoplasmic molecules are present at such high concentration that they force DNA into a small volume. This packing level requires no specific DNA-compacting protein and therefore accommodates the apparent absence of nucleosome-like particles in eubacteria. A second level of packing is represented by DNA bends and loops stabilized by the small DNA-compacting proteins. Larger proteins, such as MukBEK, may also constrain loops. Some of these proteins are likely to be displaced when a segment of DNA encounters the replication apparatus or transcription complexes. DNA looping generated by plectonemic supercoils represents a third level of compaction. The fourth level is represented by macrodomains. These large (1000 kbp), contiguous regions appear by some assays to be independent units. *E. coli* contains four macrodomains (Ori, Ter, Left, Right) and two less-structured regions. The Ori and Ter macrodomains were initially recognized by colocalization of fluorescent probes binding at a variety of map positions near *oriC* or near the terminus of replication. However, years earlier it had been noticed that chromosomal DNA contains boundaries across which DNA inversion rarely occurs. These boundaries define the macrodomains genetically: a much lower frequency of site-specific recombination occurs between sites located in different macrodomains than within the same macrodomain. What causes regions of DNA within a macrodomain to interact more with each other than with other regions is unknown.

We envision that chromosomal activities involving bulky protein complexes occur at the edges rather than in the center of the nucleoid. For example, the replication apparatus, which is likely to be attached to a multienzyme complex that supplies deoxyribonucleoside triphosphates, may be situated at the edge of the compacted portion of the chromosome, which would allow replication proteins to bind to the cell membrane. Likewise, transcription, which in bacteria is coupled to translation, also probably occurs on DNA emerging from the compacted mass of nucleoid DNA, because ribosomes are seen only outside the nucleoid (extrachromosomal localization is especially likely when transcription–translation complexes are bound to the cell membrane via nascent membrane proteins). Consistent with this idea, pulse-labeled nascent RNA is preferentially located at the nucleoid border, as is topoisomerase I (as pointed out above, topoisomerase I may serve as a cytological marker for transcription, since it is probably localized behind transcription complexes to prevent excess negative supercoils from accumulating). In special cases, such as transcription of ribosomal RNA during periods of rapid growth, transcription ‘factories’ pull together genes from different regions of the nucleoid, thereby creating local foci.

If the replication and transcription–translation machineries are located on the surface of the nucleoid, DNA movement must occur to allow access to all nucleotide sequences. Such movement may not fully explain transcriptional access to the whole genome, since some genes can be induced when DNA replication is not occurring. Compacted DNA may be sufficiently fluid that genes frequently pass from interior to exterior without guidance from proteins. At any given moment, in some fraction of the cell population each gene may be at the surface of the nucleoid and available for transcription. Capture of a gene by the transcription–translation apparatus would hold that gene on the surface. During induction of transcription, the fraction of cells in which a particular gene is captured would increase until most cells express that gene. For the chromosome as a whole, many genes would be expressing protein during active growth, and many regions would be held outside the nucleoid core. Kellenberger suggested that such activity explains why the nucleoid appears more compact when protein synthesis is experimentally interrupted.

Capture of the *oriC* region by the replication apparatus might be similar to gene capture for transcription. With the fluid chromosome hypothesis, replication proteins would assemble at *oriC* and move *oriC* from its polar position toward the midcell location of the replication apparatus. As *oriC* and nearby regions are passed through the replication forks and then replicated, new binding sites (*parS*) for the ParB chromosome partition protein would be created. Once these sites were filled, the two daughter *oriC* regions might pair through ParB–ParB interactions and return to the polar position. Other proteins would later disrupt ParB–ParB interactions, allowing the new ParB–*oriC* complex to move to the other pole of the nucleoid.

Evidence for an *oriC*-pulling force has been obtained with *V. cholerae*. In this bacterium ParB interacts with a centromere-like site (*parS*) located near *oriC*. During segregation, *parS* pulls away from nearby chromosomal loci. Meanwhile, ParA, an ATPase capable of forming filamentous polymers *in vitro*, forms a band that extends from the distant cell pole to the segregating ParB/*parS* complex. The ParA band then appears to retract, pulling the ParB/*parS* complex and the nearby *oriC* region. Whether a similar phenomenon occurs in other bacteria and whether conclusions derived from other bacteria apply to *V. cholerae* have yet to be established.

Concluding Remarks

Many of the features found in bacterial chromosomes are remarkably similar to those in eukaryotic chromosomes: one or more dissimilar chromosomes (the number can be as high as four among bacteria and as low as one in eukaryotes ($2N = 2$)), high ploidy (copy number) levels, and, at least in some species, a mitotic-like apparatus used in cell division. Consequently, the prevalent belief that profound differences exist between prokaryotic and eukaryotic chromosomes is eroding. Even the distinction revolving around histones and their compaction of DNA into nucleosomes has exceptions. True bacteria lack histones and nucleosomes, and so DNA compaction must occur by other means. But some archaea have histones and stable nucleosomes, while some unicellular eukaryotes lack both. Nevertheless, the absence of a nuclear membrane and the segregation of actively replicating bacterial chromosomes are distinct. So is the idea of a pan-genome. Genomic sequencing using DNA from multiple strains of the same bacterial species reveals that with some species individual strains can share a core genome but the overall gene content differs from one strain to another. Thus the nucleotide sequence of the total genome of some species can be far greater than that found in any given strain. To our knowledge this phenomenon has not been reported with eukaryotic organisms. One of the next tasks will be to determine whether the core sequences are physically clustered on chromosomes.

Acknowledgments

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Conjugation, Bacterial

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Glossary

coupling protein An ATPase responsible for the transport of DNA during conjugation. It is a hallmark of conjugative systems and homologues are widely distributed throughout nature.

ICE (integrating conjugative element) Chromosomally encoded elements, similar to conjugative transposons, capable of excision, conjugation, and reestablishment in a new host via integration. The excision and integration operations are formally similar to those of integrative phages.

plasmid An extrachromosomal DNA segment, usually circular, which is capable of autonomous replication via a segment of the plasmid called the replicon.

relaxase The protein responsible for site-specific nicking at the origin of transfer (*oriT*) in the DNA as well as recircularization after transfer. It covalently attaches to the 5′ end of the nicked DNA via a tyrosine. It is a key component of the relaxosome.

transconjugant A general term for a recipient cell that has successfully been converted to donor cell by conjugation.

transposon A segment of DNA that is replicated as part of a chromosome or plasmid. It encodes a mechanism, called transposition, for moving from one location to another, leaving a copy at both sites.

type IV secretion system (T4SS) A widely distributed mechanism for the secretion and uptake of protein and nucleic acids via secretion, conjugation, and transformation.

Abbreviations

Cma	chromosome mobilization ability
Eex	entry exclusion
fi/Fin	fertility inhibition
Hfr	high frequency of recombination
HFT	high frequency of transfer
HGT	horizontal gene transfer
HSL	homoserine lactone-like
ICEs	integrating conjugative elements
IHF	integration host factor
Inc	incompatibility groups
kb	kilobases
LPS	lipopolysaccharide
Mpf	mating pair formation
Mps	mating pair stabilization
NLS	nuclear localization signals
T4SS	type IV secretion system
Tc	tetracycline

Defining Statement

Bacterial conjugation is a widespread mechanism for the transfer of DNA between cells in close contact with one another. This entry summarizes past findings and discusses the better-studied systems in Gram-negative and -positive bacteria as well as the phenomena of mobilization and tumorigenesis in plants, which are related processes.

Introduction

Bacterial conjugation was first described by Lederberg and Tatum in 1946 as a phenomenon involving the exchange of markers between closely related strains of *Escherichia coli*. The agent responsible for this process was later found to be a site on the chromosome called the F (‘fertility’) factor. This finding was the basis of bacterial genetics in the 1940s and 1950s and was used extensively in mapping the *E. coli* chromosome, making it the preeminent prokaryotic organism at that time. It was also shown that F could excise out of the chromosome and exist as an extrachromosomal element or plasmid. It was capable of self-transfer to other bacteria and could cotransfer the chromosome, a serendipitous function of F, and integrate randomly into its host's DNA. The F sex factor of *E. coli* also imparted sensitivity to bacteriophages that required the F pilus, which is encoded by the F transfer region, as an attachment site during infection. In the 1960s a number of other conjugative plasmids were isolated, many carrying multiple antibiotic resistance markers. These plasmids were termed R ('resistance') factors and were found in many instances to repress pilus expression and conjugation by F, a process termed fertility inhibition (fi^+). The number of conjugative plasmids discovered has grown tremendously in the last few decades and includes self-transmissible plasmids isolated from Gram-negative and -positive bacteria as well as mobilizable plasmids. Conjugative transposons or integrating conjugative elements (ICEs), which move between cells using a conjugative mechanism, excise and integrate into the host chromosome via a process reminiscent of lysogenic phages; an example of a conjugative phage has been described for *Staphylococcus aureus*.

In general, the transfer and replication functions of these mobile elements are often physically linked and the type of transfer system is closely aligned with the nature of the replicon that is described by incompatibility groups (Inc). An excellent summary of the properties of many conjugative plasmids is given in Shapiro (1977).

Bacterial conjugation is now realized to be one of the principal conduits for horizontal gene transfer (HGT) among microorganisms. The process is extremely widespread and can occur intra- and intergenerically as well as between kingdoms (bacteria to yeast or to plants). DNA sequence analysis has revealed that conjugation, and in some cases transformation, two of the main conduits for HGT, are effected by a transenvelope protein complex that belongs to the type IV secretion system (T4SS). The effect of this process on evolution has been immense with bacteria rapidly acquiring traits both good (hydrocarbon utilization) and bad (antibiotic resistance, toxins). Once again, bacterial conjugation is at the forefront of microbiology but this time the emphasis is on the process itself rather than its utility as a geneticist's tool. Excellent reviews of the topic are provided in *The Horizontal Gene Pool, Bacterial Plasmids and Gene Spread* (C.M. Thomas, ed.) and *Plasmid Biology* (Phillips, G. and Funnell, B., eds.).

Conjugative Process

Unlike other processes like transformation and transduction that contribute to HGT, conjugation can be distinguished by two important criteria. There must be close cell-to-cell contact between the donor and recipient cells and DNA transfer must begin from a specific point on the transferred DNA molecule, be it a plasmid, transposon, or chromosome (**Figure 1**). This point is encoded within the origin of transfer (*oriT*) called *nic*. The proteins that act on this site are encoded by *tra* (transfer) or *mob* (mobilization) regions although other designations such as *vir* are now common. In general, each conjugative element encodes an array of proteins for mating pair formation (Mpf) while another set of proteins are involved in processing and transferring the DNA (Dtr). The Mpf genes can further be classified into the genes for pilus formation or mating pair stabilization (Mps) in Gram-negative bacteria or aggregate formation in

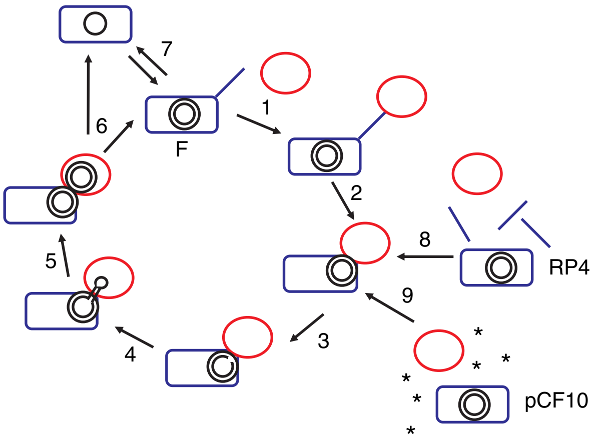

Figure 1 Summary of the mating process for universal (plasmid F) and surface-preferred (plasmid RP4) conjugation systems in Gram-negative bacteria and the pheromone-activated system of *Enterococcus faecalis* (plasmid pCF10). In universal systems, the pilus attaches to a receptor on the recipient cell surface (1) and retracts to form a stable mating pair or aggregate (2). DNA transfer is initiated (3), causing transport of a single strand in the 5′→3′ direction (4). Transfer is associated with synthesis of a replacement DNA strand in the donor cell and a complementary strand in the recipient (5). The process is terminated by disaggregation of the cells, each carrying a copy of the plasmid (6). The transfer systems of conjugative plasmids in Gram-negative bacteria can be repressed (7) or derepressed (constitutive; 8). Cells carrying RP4 and related plasmids express pili constitutively but the pili are not seen attached to the bacteria. Such cells form mating pairs by collision on a solid surface (8). In Gram-positive bacteria, such as the enterococci, the donor senses the presence of pheromone (*) released by the recipient cell, which triggers mating pair formation (Mpf) and DNA transfer (9). Donor cells are shown as oblongs (blue) and recipient (red) cells as ovals. Pili are blue.

Gram-positive cocci. A system to prevent close contact between equivalent donor cells is called surface exclusion. The gene products that process the DNA in preparation for transfer usually include a protein (relaxase) that cleaves the DNA in a sequence- and strand-specific manner at *nic* and remains covalently bound to the 5′ end in all cases that have been examined. This nucleoprotein complex plus other auxiliary proteins bound to the *oriT* region is called the relaxosome whereas the complex formed between the relaxosome and the transport machinery is known as the transferosome. A hallmark of conjugative systems is the coupling protein, within the cytoplasmic membrane, that connects the relaxosome to the transferosome. A process that prevents the transfer of DNA into the recipient cell after Mpf has occurred is called entry exclusion (Eex). Previously, the terms surface exclusion and entry exclusion were used interchangeably; however, as the details of the process have been refined, it is important to make this distinction.

In Gram-negative bacteria, the process of DNA transfer is triggered upon cell contact whereas in *Enterococcus faecalis* and T-DNA transport by *Agrobacterium tumefaciens*, among others, contact between cells induces a complex program of gene expression leading to DNA transport. Whereas the sequences for a number of conjugative elements have been completed and comparisons have revealed information on the evolution of conjugative elements, a study of the conjugative process has only been undertaken in some depth for IncF, IncI, IncP, IncW elements and the Ti plasmid of *A. tumefaciens* and other Gram-negative bacteria and for the pheromone-responsive system found in some plasmids in *Ec. faecalis*, although studies on other systems such as pIP501 are ongoing. Information is now available on the integration and excision processes of conjugative transposons and ICEs as well as the role of the *mob* genes in mobilizable plasmids. In addition, conjugation in *Streptomyces* has been studied in some detail but is quite different than that described and may use a DNA transport mechanism related to the process of DNA partition during septation in *Bacillus subtilis* (see '*Streptomyces*').

Physiological Factors

The level of transfer efficiency varies dramatically among the various systems. For derepressed or constitutively expressed systems such as F (IncFI) or RP4 (IncPα), maximal levels of mating (100% conversion to plasmid-bearing status) are possible within 30 min. Plasmids undergoing fertility inhibition usually have a 100- to 1000-fold reduction in mating efficiency whereas other plasmids, especially the smaller plasmids of Gram-positive bacteria and conjugative transposons, mate at barely detectable levels even under the best of circumstances. Factors affecting mating efficiency include temperature with very precise optimums usually being the rule. For instance, F and RP4 mate optimally at 37–42 °C, and IncH plasmids and the Ti plasmid at about 20–30 °C. Other factors include oxygen levels, nutrient availability, and growth phase. Silencing by host-encoded factors such as H-NS is an important phenomenon that is thought to provide control of gene expression by newly acquired DNA through HGT, a process now termed 'xenogeneic silencing'. F^+ cells in late stationary phase are known as F^- phenocopies because they are able to accept incoming F DNA and are not subject to surface or entry exclusion. Available literature indicates conjugation to be maximal over a short temperature range, in nutrient-rich environments with good aeration for aerobic organisms.

Liquid versus Solid Support

The ability of some conjugative systems to mate equally well in liquid media or on a solid support is one of the hallmarks of conjugation. Whereas all conjugative elements can mate well on a solid support, usually a filter placed on the surface of a prewarmed nutrient agar plate, many transfer systems, including those of the IncF group and the pheromone-responsive plasmids of *Enterococcus*, mate very efficiently in liquid media. This difference can be attributed to the nature of the Mpf process as thick, flexible pili of Gram-negative bacteria are associated with systems that mate well in liquid media whereas rigid pili, not usually seen attached to the cells (e.g., IncPα), require a solid support for efficient mating. The aggregation substance of *Ec. faecalis* allows high levels of transfer in liquid media but other Gram-positive systems and conjugative transposons mate at low levels and absolutely require a solid support. In general, it appears that mating systems requiring a solid support depend on collision between donor and recipient cells whereas systems that mate well on either medium have a mechanism for initiating contact between freely swimming cells (thick, flexible pili, and aggregation substance). The description of media requirements for many Gram-negative plasmid transfer systems is given in Bradley *et al.* (1980).

Conjugative Elements

Naturally occurring conjugative elements including plasmids, conjugative transposons, or ICEs, which are incorporated into the host chromosome, can lead to chromosome mobilization ability (Cma), resulting in high frequency of recombination (Hfr). Free plasmids can be divided into self-transmissible (Mpf plus Dtr genes) or mobilizable (Dtr or Mob genes) plasmids and can vary in size from a few kilobases (kb) to large plasmids 100–500 kb in size.

Plasmids

In general, Gram-negative transfer systems are approximately 20–35 kb and reside on plasmids from 60 to 500 kb whereas mobilizable plasmids are under 15 kb. The transfer or mobilization regions often represent half or more of the coding capability of the plasmid. **Table 1** contains a list of selected plasmids and their characteristics including their pilus type and mating medium preference. In nonfilamentous Gram-positive plasmids, the smaller plasmids (<30 kb) usually have a requirement for a solid support during mating and mate at low levels whereas the larger plasmids mate efficiently in liquid media and express genes for aggregate formation (e.g., *Enterococcus*, *Staphylococcus*, *Lactobacillus*, *Bacillus thuringiensis*). *Streptomyces* is able to mate at high frequency and has the added property of Cma. Each large self-transmissible plasmid can supply the needed Mpf functions for a number of mobilizable plasmids. These mobilizable plasmids have been used to construct vectors that either are maintained in the recipient cell or deliver their cargo of DNA but are unable to replicate in the new host (suicide vectors). This has been a boon for the study of genetics of otherwise recalcitrant bacteria.

Chromosome Mobilization

F undergoes integration into the chromosome via four transposable elements (IS2, IS3a and b, and Tn1000), which either mediate cointegrate formation via a transposition event or more frequently undergo homologous recombination between these sequences and similar elements on the chromosome. Once incorporated into the chromosome, the F replicon is suppressed by the chromosomal replication machinery allowing stable maintenance of the Hfr strain. Like F, which was found incorporated into the host chromosome, other examples of naturally occurring Hfr strains have been reported in the literature including ICEs. Hfr strains have also been constructed using homologous gene segments shared by a

Table 1 Selected conjugative/mobilizable plasmids and conjugative transposons

Mobile element	*Size (kb)*	*Inc group/host/pheromone*[a]	*Copy number*	*Mating surface/pilus type*[b]	*Mating efficiency/host range*
Gram-negative bacteria					
F	100	IncFI	1–2	Liquid/flexible (II)	High (derepressed)/narrow
RP4	60	IncPα	4–6	Solid/rigid (II)	High (constitutive)/broad
ColIB-P9	93	IncI1	1–2	Liquid/rigid (II), thin (IV)	Low (repressed)/narrow
pTiC58	~200	*Agrobacterium tumefaciens*/HSL	1–2	Solid/rigid (II)	Low (repressed)/narrow
vir	25 (T-DNA)	Plant exudates	1–2	Plants/rigid (II)	
Gram-positive bacteria					
pAD1	60	*Ec. faecalis*/cAD1	1–4	Liquid	High (10^{-2} per donor)/narrow
pIP501	30.2	Inc18/*Streptococcus agalactiae*	3–5	Solid	High (10^{-4} per donor)/broad
pIJ101	8.8	*Streptomyces*	~300	Solid	High/broad (*Actinomycetes*)
Mobilizable plasmids					
ColE1	6.6	*Escherichia coli*	~10	Liquid	High (IncF, -P, -I)/narrow
RSF1010	8.9	IncQ	~10	Solid	High (IncP)/broad
pMV158	5.5	*Streptococcus* B	12–16	Solid	Low (pAMβ1/pIP501)/broad
Conjugative transposons					
Tn*916*	18.5	*Enterococcus faecalis*		Solid	Low (~10^{-8} per donor)/broad
CTnDOT	80	*Bacteroides*/Tc		Solid	Low (~10^{-5} per donor)/narrow
Integrating conjugative elements					
SXT	99.5	*Vibrio cholerae*		Solid	Low (~10^{-4} or 10^{-5} per donor)/narrow
R391	89	*Providencia rettgeri*		Liquid	Low (~10^{-4} or 10^{-5} per donor)/narrow

[a] Incompatibility groups (Inc) are listed for *E. coli* except pIP501, which uses the Inc group classification for *Streptococcus.* HSL is homoserine lactone. cAD1 is a pheromone specific for pAD1 of *Ec. faecalis.* CTnDOT transfers at 1000-fold higher frequencies in the presence of tetracycline (Tc).
[b] Pili can be classified as type II or type IV that are assembled by type II (T2SS) and type IV (T4SS) secretion systems, respectively.

plasmid and its host for mapping the host's genome. Since the advent of pulsed-field gel electrophoresis for mapping chromosomes and large-scale sequencing facilities, the utility of Hfr strains for mapping purposes is waning. The procedure for using Hfr strains for chromosome mapping requires that the plasmid integrate near a locus with a few genetically defined markers. The direction of transfer of the chromosome and the time of entry of the markers into the recipient cell is a function of the position and orientation of the plasmid's *oriT* in the chromosome and its distance from each marker. By laboriously measuring the time of entry of each marker (e.g., antibiotic resistance, amino acid biosynthesis), which must be able to recombine into the recipient's chromosome in such a way as to announce its presence, a map of the chromosome can be generated. The process of mobilizing the entire *E. coli* chromosome takes about 90 min and markers that are distal from F *oriT* are transferred much less efficiently than those more proximally located, a process called the 'gradient of transmission'. The last portion of the chromosome to be transferred contains the F transfer region; consequently, the recipient cells in an Hfr mating are seldom, if ever, converted to F^+ (Hfr) status.

Another related property of F is imprecise excision out of the chromosome with adjoining chromosomal sequences being incorporated into the circular F element that are often large enough to encode complete operons. These elements are known as F′ (F prime) factors, and examples include F*lac*, F*gal*, and F*his*.

Conjugative Transposons and ICEs

The first conjugative transposon, Tn*916*, which expressed *tetM* (tetracycline (Tc)) resistance, was isolated from *Ec. faecalis* (**Table 1**) in Clewell's laboratory in 1981. This element excises from the chromosome, circularizes into a nonreplicative intermediate, and expresses functions for transfer to a wide range of recipient cell types at low frequency on solid surfaces. The enzymes responsible for excision and integration (Int, Xis) are related to the corresponding enzymes in lambda (λ) phage. The absence of a small repeated sequence flanking the conjugative transposon as well as a nonrandom site selection mechanism for integration suggests that these elements are evolutionarily more related to phages than true transposons. Excision of the element results in staggered ends that form a heteroduplex structure upon circularization. This heteroduplex structure is derived from the flanking sequences in the chromosome ('coupling sequences'). On the basis of mapping and sequencing the site of insertion and determining which end of the heteroduplex is inherited in the recipient, a model involving single-stranded DNA transfer has been proposed. A second important group of conjugative transposons has been found in the anaerobic Gram-negative genus *Bacteroides*. These elements are usually associated with antibiotic resistance (*tetX, erm*) and exhibit increased transfer proficiency in the presence of Tc. That IncJ conjugative plasmids could not be isolated from *E. coli* was puzzling. The realization that they were related to the chromosomally located, conjugative, integrative phage-like SXT element in *Vibrio cholerae* explained why they were not true plasmids. These ICEs appear to lack the property of random site selection during integration but instead integrate at *att*-like sites (e.g., *prfC*), similar to lysogenic phages.

Conjugative transposons demonstrate amazing versatility in mobilizing DNA. They can mobilize coresident plasmids directly or form cointegrates with plasmids or the chromosome. They are also able to harbor other mobile elements such as transposons and move them between cells. In the case of *Bacteroides*, the conjugative transposons are able to excise and mobilize small, nonconjugative, nonreplicative segments of DNA found in the chromosome called NBUs (nonreplicating *Bacteroides* units). While conjugative transposons have been identified in many genera of bacteria especially in Gram-positive bacteria, the details of the conjugation process remain obscure although orthologues of T4SS have been identified within the transfer regions, suggesting that Gram-negative and -positive conjugation are related.

Gram-Negative Conjugation

With the possible exception of *Bacteroides*, all Gram-negative transfer systems encode a conjugative pilus (type II), which is essential for Mpf and DNA transport (**Figure 1**). In thick, flexible pilus mating systems, the pili are attached to the donor cell and pili-mediated contact between donor and recipient cells is easily visualized with a electron microscope. In the case of rigid pilus mating systems, pili are rarely seen attached to cells but are seen as bundles of pili accumulating in the medium.

Considerable homology has been found among all the transfer systems examined to date within the Gram-negative group of organisms as well as detectable homology with systems in Gram-positive bacteria and Archaea. In Gram-negative bacteria, two broad classes can be identified as F-like or P-like Mpf, with I-like Mpf systems being a subclass of P. Relaxase, as well as the protein that energizes DNA transport, the coupling protein, is conserved throughout these systems as are key proteins in the T4SS, as described below. To date, the principal systems studied include IncF (F, R100-1, R1) and IncHI1, which have F-like Mpf genes, and IncP (RP4, R751), IncI1 (ColIb-P9, R64), IncN (pCU1, pKM101), IncW (R388), and the Ti plasmids of *A. tumefaciens*, which encode IncP-like Mpf genes.

Pilus

Structure

The conjugative pilus is a thin filament expressed in relatively low numbers (1–3 per cell), has no set length (usually ~1 μm), and is randomly distributed on the surface of the cell (**Figure 2**). The diameter of the pilus is approximately 6–11 nm, with most pili being around 9 nm. Pili isolated from cells usually contain a 'knob' at the base that represents unassembled pilin subunits derived from the inner membrane of the cell. They may also have a pointed tip suggesting another protein or unusual configuration of pilin subunits at the tip and can aggregate into large bundles that can be pelleted in an ultracentrifuge. The pilus is usually composed of a single

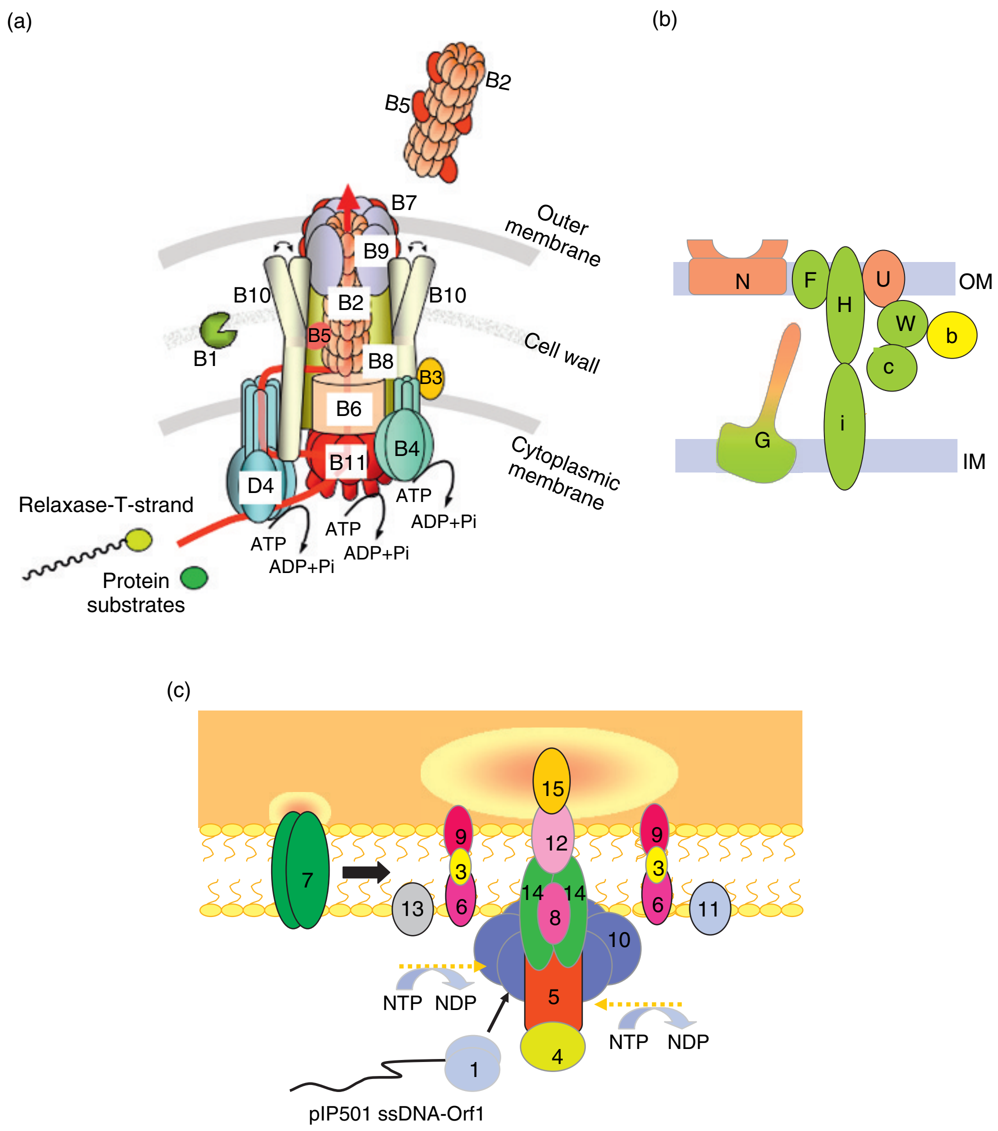
(a)
B2
B5
B7
Outer membrane
B9
B10
B2
B10
B1
B5
B8
Cell wall
B3
B6
B4
B11
D4
Cytoplasmic membrane
Relaxase-T-strand
ATP
ADP+Pi
ATP
ADP+Pi
ATP
ADP+Pi
Protein substrates
(b)
N
F
H
U
OM
W
b
c
G
i
IM
(c)
15
9
12
9
7
3
3
13
6
14
14
8
6
11
10
NTP
NDP
5
NTP
NDP
4
1
pIP501 ssDNA-Orf1

Figure 2 Transfer apparatuses of Gram-positive and -negative bacteria. The simplest transfer mechanism is exemplified by Tra in *Streptomyces*, which is homologous to the coupling protein of other systems (not shown). (a) The transfer apparatus of the Ti plasmid illustrates the complexity of the P-type Gram-negative transfer systems. The red arrow is the putative path of the DNA during transfer. Note the presence of three ATPases involved in pilus assembly and DNA transfer. Reprinted with permission from AAAS. (b) The F-type Gram-negative system is similar to that for the Ti plasmid except that it lacks homologues of VirB11 (ATPase) and VirB8. It contains proteins for mating pair stabilization (Mps) (TraG, -N, and -U in orange) as well as a cluster of proteins involved in pilus assembly that form an interaction network (shown in green) (based on Harris and Silverman (2004) *Journal of Bacteriology.* 186: 5480–5485). TrbB (yellow) has DsbC-like activity; TraN, -U, and -H are cysteine-rich. TraD, the coupling protein, is not required for pilus assembly. The relaxase, other members of the relaxosome, and inessential proteins are not shown. (c) The transfer apparatus of pIP501 illustrates the comparative simplicity of a system that spans only one membrane. Homologies among the various systems as well as functions are given in **Table 2**. From Abajy *et al.* (2007) *Journal of Bacteriology* 189: 2487–2496, redrawn with permission.

repeating subunit of pilin arranged in a helical array with a hollow lumen clearly visible in negatively stained electron micrographs. F pili, which are the best studied, have a diameter of 8 nm with an inner lumen of 2 nm and a mass per unit length of 3000 Da Å^{-1}. The pilin subunits are arranged as repeating layers of five subunits with each layer rising 1.28 nm.

The F pilus is expressed as propilin of 121 amino acids (*traA*), which requires TraQ, a putative chaperone, for insertion in the inner membrane where it is stored in a pool of approximately 100 000 subunits. The 51-amino acid leader peptide is cleaved by the host leader peptidase (LepI) and the pilin subunit is acetylated at the N-terminus by TraX. Transposon insertion studies have revealed that mature pilin is oriented within the inner membrane as two α-helical transmembrane segments with the N- and C-termini facing the periplasm. Assembly by the TraL, -E, -K, -B, -V, -C, -W, -U, -F, -H, -G and TrbC proteins results in the subunits oriented within the fiber such that the acetylated N-terminus is buried within the structure and the C-terminus is exposed on the sides. The pilus appears to assemble at its base rather than the tip, based on evidence using the slowly assembled pili expressed by the IncHI1 plasmid R27.

The RP4 pilin subunit is expressed as a 15 kDa prepilin polypeptide (*trbC* in Tra2), which is processed three times to give a 7.5 kDa mature product that is circular with the N- and C-termini covalently linked. The cleavage reactions at the N- and C-termini are completed by LepI of the host as well as the cyclase, TraF, that removes four amino acids at the C-terminus and cyclizes the pilin. The RP4 transfer region is separated into two parts: Tra1 and -2. In addition to *traF* of Tra1, an essential gene, *trbD-L* are required for pilus assembly with the exception of TrbK, which is involved in Eex. A homologue of F TraX is present in RP4 (TraP) although its substrate and function are unknown. Circular pilins have been identified in the IncHI1 plasmid, R27, and T-pili of the Ti plasmid of *A. tumefaciens*, that lacks a plasmid-encoded cyclase, and are suspected to be present in other mating systems that encode a P-like TraF orthologue.

Phage attachment

Conjugative pili act as the primary receptor for a wide range of bacteriophages. These phages can be divided broadly into those that bind to the pilus tip and those that bind to the sides of the pilus. The structure of the phages includes the single-stranded DNA filamentous phages, the small isometric RNA phages, and the complex double-stranded DNA tailed phages that usually attach near the pilus tip. The filamentous phages specific for F-like pili (Ff phages; M13, f1, fd) attach via a defined region of the pIII attachment protein to an unknown receptor at the pilus tip. The RNA phages such as R17 and Qβ, which belong to different phage groups, bind to specific residues in F pilin exposed on the pilus sides. For RP4 (IncPα), the filamentous phage Pf3 binds to the sides of the pilus as does the RNA phage PRR1. The tailed phages such as PRD1 and PR4 bind to the pilus tip, although it appears that infection requires binding to a tip exposed at the cell surface rather than at the end of an extended pilus. These phage have a broad host range including cells bearing IncP, -W, -N, and -I plasmids.

Whereas the pilus is required for initial attachment, the transfer region is not necessarily required for phage penetration or growth. The Ff phages are thought to contact the cell surface via the process of pilus retraction where they interact with the TolA protein and penetrate the cell via the TolQRA pathway. The RNA phages R17 and Qβ have differing requirements for the F TraD coupling protein, which energizes the transport of nucleic acid through the conjugation pore, suggesting that R17 is imported via the F transfer machinery whereas Qβ is taken up via another pathway. RNA phages have the property of phage eclipse, an extracellular event that involves detachment of the phage capsid from the attachment (A) protein–RNA complex, which is then susceptible to exogenous ribonuclease. This process requires that the pilus be extended from a living cell, suggesting that the cell supplies the energy for phage eclipse in some unknown manner. Mutations in F-pilin that block phage eclipse have been identified.

Role in conjugation

The role of the pilus in conjugation has been controversial but it is now generally believed that pilin is part of the conjugative pore. Mutations that affect pilus formation block both Mpf and DNA transfer. Mutations that affect Mps (e.g., F TraN and TraG) allow initial contacts to form between cells via the pilus and also allow the initiation of DNA synthesis in the donor cell but block DNA transport into the recipient cell. While indirect, this is the best evidence that the pilus is involved in the signaling process whereas the Mps genes are involved in forming the conjugation pore. Other experiments in which donor and recipient cells were not allowed to establish cell-to-cell contact suggested that mating was possible through the extended pilus. The pilus has also been implicated in DNA transport in both the F plasmid (mutations in pilin that block transfer) and the Ti plasmid (cross-linking T-DNA to pilin).

Mating Pair Formation

Conjugation involves the establishment of a mating pair via Mpf, which may be augmented by the process of Mps, a feature of F-like systems. The pilus is thought to identify a receptor on the recipient cell surface that triggers retraction of the pilus into the donor cell, although the route of the pilin subunits in this process is unknown.

Pilus outgrowth requires energy whereas retraction occurs by default in the absence of assembly. Thus factors that negatively affect cell metabolism (temperature, poisons, and carbon source) cause retraction. Whether pilus outgrowth and retraction are ongoing processes or whether binding of recipient cells or phage trigger retraction is unknown.

Early studies identifying mutations in the recipient cell that affected conjugation (Con^{-}) revealed that various components of the heptose-containing inner core of the lipopolysaccharide (LPS) were generally important in Mpf whereas OmpA was required for efficient conjugation by the F transfer system. The F-like systems are each affected by different mutations in the *rfa* (now *wra*) locus in the recipient cell whereas the IncH plasmids seem to recognize a generalized negative charge on the recipient cell surface. The requirements for OmpA by F as well as for specific side chains in the LPS appear to be a function of the outer membrane protein F TraN, which is involved in Mps. The idea that the pilus recognizes negatively charged surfaces nonspecifically remains a possibility. A second protein identified in F that is involved in Mps is TraG. Mutations in *traG* fall into two classes: those in the first two-thirds of TraG that affect pilus formation and Mpf and those that only affect Mps.

Recent studies on the F-like *tra* and Ti *vir* gene products have revealed the presence of a complex transenvelope structure, the transferosome, composed of core T4SS proteins and auxiliary proteins involved in pilus retraction and Mps (**Figures 2**(**a**) and **2**(**b**); **Table 2**). The scaffold for the transferosome consists of a TraB (VirB10)–TraK (VirB9)–TraV (VirB7) complex that spans the envelope. VirB10 has been shown to have TonB-like activity that transduces energy from the inner membrane to the outer membrane; TraK/VirB9 are related to secretins of other secretion systems and TraV/VirB7 are lipoproteins in the outer membrane. Other transfer proteins are also required for pilus assembly (**Figure 2**(**b**)) with a group of proteins (TraF, -G, -H, -N, -U, -W, TrbB, -C, and -I), which are characteristic of F-like transfer systems, additionally involved in pilus retraction and conjugative pore formation. Some of these proteins have a high cysteine content (TraH, -N, and -U) whereas others are homologous to thioredoxin with one protein (TrbB, a homologue of DsbC) shown to be involved in disulfide bond formation and protein stabilization.

An interesting variation of Mps for the IncI1 transfer systems (R64, ColIb-P9) that express two types of pili has been identified: thin, flexible pili, which are required for Mps, and thick, rigid pili, which are required for DNA transfer. Research on the thin pili of R64 by Komano's group has revealed that they are composed of type IV pilin (similar to the pili found in pathogens such as *Neisseria gonorrhoeae*) of 15 kDa (*pilS*). These pili have a protein at their tip (*pilV*) whose gene undergoes rearrangement via site-specific recombination by the *rci* gene product to form seven possible fusion proteins, each recognizing a specific LPS structure (e.g., *pilVA′*). Whether these pili retract in order to bring the donor and recipient cells together is unknown although retraction is a general feature of type IV pili. Besides these two cases (F and R64 thin pili), little is known about Mps in other Gram-negative systems.

Table 2 Orthologues in various conjugative systems[a]

Function	*F*[b]	*P(RP4)*	*Ti(pTiC58)*	*I1(R64)*	*pIP501*	*pAD1*	*HI1(R27)*
SLT[c]	P19	TrbN	VirB1	TrbN	Orf7	Orf41/50	Orf169
Acetylase	TraX	TrbP					
Pilin cyclase		TraF	Unknown				TrhP
Pilin	TraA	TrbC	VirB2	TraX			TrhA
Pore, pilus assembly	TraL	TrbD	VirB3				TrhL
Pilus assembly, ATPase	TraC	TrbE	VirB4	TraU	Orf5		TrhC
Pore, pilus assembly	TraE	TrbF	VirB5				TrhE
Pilus assembly, Mps	TraG^{N}[b]	TrbL	VirB6				TrhG
Lipoprotein, OM	TraV	TrbH	VirB7	TraI			TrhV
Pore		TrbF	VirB8				
Pore (secretin-like)	TraK	TrbG	VirB9	TraN			TrhK
Pore (TonB-like)	TraB	TrbI	VirB10	TraO			TrhB
Transport ATPase		TrbB	VirB11	TraJ			
Relaxase	TraI	TraI	VirD2	NikB	Orf1(TraA)	TraX	TrhI
Coupling protein ATPase	TraD	TraG	VirD4	TrbC	Orf10	TraW	TraG

[a] Based on Lawley *et al.* (2003); Grohmann *et al.* (2003); Abajy *et al. J. Bacteriol*. 189: 2487–2496 (2007).

[b] F-like systems contain a characteristic gene cluster encoding mating pair stabilization (Mps), disulfide bond isomerization, and pilus retraction proteins (TraF, -H, -G, -N, -U, -V, and -W; TrbB, -C, and -I). G^{N} refers to the N-terminal region of TraG that is required for both pilus assembly and Mps.

[c] SLT refers to soluble lytic transglycosylase.

Surface and entry exclusions

Surface or entry exclusion reduces redundant transfer between equivalent donor cells. Such transfer is thought to be deleterious to the donor cell and is exemplified by the phenomenon of lethal zygosis, which occurs when a high ratio of Hfr donor to recipient cells is used. Multiple matings with a single recipient cell result in its death because of severe membrane and peptidoglycan damage as well as induction of the SOS response resulting from the influx of a large amount of single-stranded DNA. The surface exclusion genes were first identified as the *ilz* locus (immunity to lethal zygosis) because of their role in protecting recipient cells during matings with a high ratio of Hfr donor to recipient cells.

The mechanism of entry or surface exclusion is unknown although an exclusion mechanism has usually been found associated with the transfer systems studied to date. One exception is the conjugative transposons that transfer at such low frequency that redundant transfer might not be an important factor. Surface exclusion in the F system involves TraT, a lipoprotein found in the outer membrane, which forms a pentameric structure and blocks Mps. Whether it interacts with the pilus or another component of the F transfer system is unknown. The TraS protein of F is an inner membrane protein that blocks the signal that DNA transfer should begin and is thus associated with the property of Eex. TraS interacts with TraG in the inner membrane of another donor cell as demonstrated by Eex specificity experiments using F and R100 plasmids and R391 and SXT ICEs. Thus, TraG appears to contact the inner membrane of the recipient cell, thereby stapling the cells together as part of the Mps process, which is blocked by TraS. TrbK, a lipoprotein found in the inner membrane of RP4-containing cells, is also thought to cause Eex.

DNA Metabolism

Organization of oriT

In Gram-negative transfer systems, the origin of transfer (*oriT*) is ~40–500 bp in length and contains intrinsic bends and direct and inverted repeats that bind the proteins involved in DNA transfer. The *nic* site itself, which is a strand- and sequence-specific cleavage site, is cleaved and religated by relaxase. In most cases, relaxase requires auxiliary proteins that direct relaxase to the *nic* site and ensure the specificity of the reaction. The sequence of the *nic* sites identified to date reveal four possible sequences represented by IncF, -P, and -Q and certain Gram-positive plasmids such as pMV158. In addition, there is usually a protein that binds to multiple sites within *oriT* forming a higher-order structure in the DNA, which is essential for the process. This protein also appears to have a function in anchoring the relaxosome to the transport machinery.

Mechanism of DNA transfer

After Mpf, a signal is generated that converts the relaxosome from the cleavage/religation mode to one where unwinding of the DNA is coupled to transport through the conjugation pore in an ATP-dependent manner. The transfer rate is ~750 nucleotides per second with the F plasmid (100 kb) transferred in a little over 2 min.

In IncF plasmids, TraI is the relaxase/helicase enzyme that binds to a site near *nic* and generates an equilibrium between cleavage and religation. This reaction requires supercoiled template DNA and Mg^{2+} as well as the auxiliary proteins F TraY and host integration host factor (IHF) *in vitro*. TraM is known to promote nicking although it is not absolutely required. The signal that triggers the helicase activity of F TraI, which is essential for DNA transfer, is unknown as is the function of TraI* produced by a translational restart in the *traI* mRNA. TraY binds near *nic* whereas TraM binds to multiple sites, in conjunction with IHF, within the nucleoprotein complex. TraM binds TraI as well as the coupling protein, TraD, an inner membrane protein that utilizes ATP via its two NTP-binding motifs. Thus TraM could mediate the initial interaction between TraI and TraD in F, whereas in other systems, relaxase and coupling protein interact directly. This step precedes transport of relaxase, covalently bound to the 5′ end of the DNA, into the recipient cell where religation is thought to occur. The transport of relaxase has been demonstrated for several systems other than F and is now thought to be a general feature of conjugation.

In RP4, a similar arrangement of proteins at *oriT* exists except that there is no role for the host protein, IHF. The relaxase protein, also called TraI, cleaves at *nic* and is part of a complex with TraJ whereas TraH stabilizes the TraI–TraJ complex. TraK binds and bends the DNA at *oriT* to form the nucleosome-like structure thought to be needed to initiate DNA replication. The DNA is delivered to the TraG coupling protein, possibly by another ATPase, specific to P-like systems, called TrbB in RP4 and VirB11 in the Ti plasmids, found in the inner membrane.

In all cases, the 5′ phosphate generated by the cleavage reaction remains covalently bound to the relaxase enzyme via a tyrosine residue using a mechanism that is similar to the initiation of rolling circle replication in some phage and plasmid replicons. The DNA is transferred in a $5' \rightarrow 3'$ direction with the first genes to enter the recipient cell called the leading region. Transfer seems to be a precise process with termination of transfer after one copy of the plasmid has been delivered to the recipient cell. A sequence in *oriT*, near *nic*, is important for termination by relaxase in a religation reaction. Both strands of the DNA are replicated by the PolIII enzyme using discontinuous synthesis in the recipient and continuous synthesis either from the free 3′ end at *nic* or from an RNA primer in the donor. Although synthesis and transport are coupled in conjugation, DNA synthesis does not drive, nor is it required, for DNA transfer.

In RP4 and IncI plasmids, the transport of a primase protein, Pri (*traC* encoded in Tra1), or Sog in RP4 or IncI plasmids, respectively, has been demonstrated to occur simultaneously with the transport of the DNA, with hundreds of copies being transferred. This protein appears to initiate DNA synthesis in the recipient cell via primer formation although it is not essential for conjugation. In F, no primase is transferred and DNA synthesis is thought to begin via a mechanism utilizing *ssi* sites for single-stranded initiation.

Leading region expression

The first genes to enter the recipient cell in the leading region include genes for preventing the SOS response (*psi*) and for plasmid maintenance via poison–antidote systems such as CcdAB and Flm in F, Hok/Sok in R1, and Kil/Kor in RP4. Although homologues of a single-stranded DNA-binding protein, Ssb, are found on many conjugative plasmids, they are not essential for conjugation. Another interesting but inessential gene is *orf169* in F or TrbN in RP4 that is related to transglycosylases such as lysozyme. Perhaps this gene, which is the first to enter the recipient cell during F transfer, has a role in establishing a new transferosome in the recipient cell. Slowly growing bacteria in natural environments might require this transglycosylase to rearrange peptidoglycan in preparation for pilus assembly and DNA transfer. Homologues in Gram-positive conjugation systems are essential for conjugation, suggesting a role in penetrating the thick Gram-positive cell wall during erection of the transport apparatus.

Regulation

- The regulation of genes involved in conjugation has been extensively studied in F, RP4, and Ti (see ‘Transfer to plants’) plasmids and in Gram-positive conjugation but there is little information on other systems. The regulation of F transfer gene expression depends on both host and plasmid-encoded factors whereas the regulation of RP4 appears to be independent of the host. Also, F is unusual in that there is no evidence for coregulation of transfer and replication, a salient feature of other conjugation systems.

In F there are three main transcripts encoding *traM*, *traJ* (the positive regulator of transfer operon expression), and *traY-I* (33 kb). The P_{Y-X} promoter is controlled by a consortium of proteins, including the essential TraJ protein, TraY, the first gene in the operon, and IHF and SfrA (also known as ArcA) encoded by the host. TraY also controls *traM* expression from two promoters that are autoregulated by TraM. The translation of the *traJ* mRNA is controlled by an antisense RNA FinP, which requires the RNA-binding protein FinO for activity (fertility inhibition; see ‘Fertility inhibition’). Extracytoplasmic stress, which upregulates the CpxAR regulon, decreases the levels of TraJ by upregulating the HslVU protease/chaperone pair that degrades TraJ. F transfer gene expression is also silenced by H-NS as it enters the stationary phase with TraJ involved in desilencing the transfer region. This silencing has also been described for other plasmids in response to temperature flux and some plasmids such as F-like R100 encode H-NS-like proteins that form heterodimers with host H-NS, thereby inactivating it and desilencing the transfer region. Other factors have been shown to affect F transfer gene expression including LRP and CRP, which sense the nutritional state of the host cell, and Dam methylation of sites within the *traJ* promoter region during conjugation, which monitors the methylation state of the incoming DNA.

In RP4, transfer is tied very closely to replication with the main replication promoter for TrfA divergently oriented and overlapping with the first of two promoters for the Tra2 transfer region, P_{trbA} and P_{trbB}. In this system, the *trfA* promoter is activated first in the transconjugant, promoting plasmid replication. The P_{trbB} promoter, as well as the promoters in Tra1 that express the genes for Dtr, is also activated in order to establish a new transferosome. Eventually, the main global regulators KorA and KorB repress expression from these promoters and allow transcription from P_{trbA} that maintains the level of Tra2 proteins in the donor cell. TrbA is a global regulator that represses P_{trbB} as well as the three promoters in Tra1 encoding the genes for Dtr. Thus conjugation leads to a burst in transcription that establishes the plasmid in the new donor cell followed by transcription from either the *trfA* or the *trbA* promoters during vegetative growth.

Fertility inhibition

Fertility inhibition (Fin) is a widespread phenomenon among related plasmids that limits the transfer of competing plasmids coresident in a single cell. The Fin systems of F-like plasmids (R factors) repress F and also autoregulate the expression of their own transfer regions. These systems have two components, the antisense RNA FinP and the RNA-binding protein, FinO, which together prevent translation of the *traJ* mRNA, TraJ being the positive regulator of the P_{Y-I} promoter. FinO protects FinP antisense RNA from degradation by the host ribonuclease, RNase E, allowing FinP concentration to rise sufficiently to block *traJ* mRNA translation. Whereas *finP* is plasmid-specific, *finO* is not and can be supplied from a number of F-like plasmids. This is the basis of the fi^+ phenotype noted in the 1960s for various R factors. F lacks FinO since *finO* is interrupted by an IS*3* element and consequently is constitutively derepressed for transfer.

In F-like plasmids (FinOP$^+$), 0.1–1% of a repressed cell population express pili. If conjugation is initiated, the transconjugant is capable of high frequency of transfer (HFT) for about six generations until fertility inhibition by FinOP sets in. This phenomenon, along with surface or entry exclusion, contributes to the epidemic spread followed by stable maintenance of a plasmid in a natural population of bacteria.

Other Fin systems are specified by one plasmid and are directed against another. For instance, F encodes PifC that blocks RP4 transfer whereas RP4 encodes the Fiw system that blocks the transfer of coresident IncW plasmids. Each system has a unique mechanism that has made the study of Fin systems more difficult and has tended to downplay the importance of this phenomenon in the control of the dissemination of plasmids in natural populations.

Gram-Positive Conjugation

Conjugative elements in nonfilamentous Gram-positive bacteria can be subdivided into three groups: small plasmids (<30 kb), usually associated with MLS resistance, exhibiting moderate transfer efficiency on solid surfaces over a broad host range (pAMβ1, pIP501); large plasmids (>60 kb) that mate efficiently in liquid media over a narrow host range and undergo clumping or cell aggregate formation (pAD1, pCF10 in *Enterococcus*, pSK41, pGO1 in *S. aureus*); and conjugative transposons.

Studies on Gram-positive plasmids have revealed that their conjugative systems also encode homologues of T4SS proteins especially relaxase, the coupling protein (an ATPase associated with pilus assembly), and a lytic transglycosylase (Ti VirD2, -D4, -B4, -B1, respectively; **Figure 2(c)**, **Table 2**). Although constructing the T4S apparatus within the Gram-positive cell envelope has different challenges, the basic mechanism of conjugation appears to be conserved throughout the phylogenetic tree.

In the few systems studied in detail, detection of a recipient cell results in expression of an aggregation substance (AS, Agg, or Clu) that covers the surface of the donor cell and results in the formation of mating aggregates that are visible to the naked eye. This ‘fuzz’ is the result of a complex pattern of gene expression that has been studied in detail in only a few instances, mostly in the large plasmids of *Ec. faecalis*. This group of plasmids responds to pheromones expressed by the recipient cell whereas the signal that triggers mating aggregate formation in other systems is poorly understood. Plasmids in *S. aureus* produce pheromones that trigger transfer by *Ec. faecalis*, suggesting a mechanism to broaden the host range for the plasmids of this latter organism.

Enterococcus faecalis

The first conjugative plasmid identified in Gram-positive bacteria was pAD1, which carried a hemolysin/bacteriocin determinant responsible for increased virulence and which caused clumping or aggregation 30 min after the addition of plasmid-free cells. Later it was shown that aggregation and subsequent plasmid transfer were induced by pheromones produced by the recipient cell and released into the medium. These pheromones are hydrophobic peptides of 7–8 amino acids with a single hydroxyamino acid derived from the signal sequences of surface lipoproteins. Each recipient cell releases several pheromones with plasmids from a particular incompatibility group recognizing a specific pheromone with specificity residing in the N-terminus of the peptide. Once the plasmid has become established in the transconjugant, expression of the pheromone is repressed by preventing its synthesis in, or release from, the donor cell. As little as picomolar amounts of pheromone added to donor cultures can induce the expression of the transfer genes. Usually the concentration hovers around 1–10 nmol l^{-1} and a slight increase in pheromone levels is sufficient to induce clumping. Pheromones are named after the transfer system that recognizes them; thus, pAD1 recognizes cAD1 and produces iAD1, an inhibitory peptide that blocks accidental induction of transfer.

The pheromone is recognized by a plasmid-encoded protein and imported into the cell via the host Opp system (oligopeptide permease). In pAD1, it binds to a repressor, TraA, inactivating it and allowing transcription of TraE1, a positive regulator required for induction of transfer gene expression. In pCF10, the pheromone cCF10 causes antitermination of transcription of the *prg* genes (pheromone-responsive gene), generating the 530-nucleotide RNA Q_L and the mRNA for aggregation substance and transfer proteins. The Q_L RNA, in conjunction with the pheromone, associates with the ribosome causing preferential translation of the transfer genes. In either case, transcription of the aggregation substance (AS or Asa1 for pAD1, Asc10 for pCF10) ensues, which is deposited asymmetrically on the surface of the cell until the cell surface is covered. This binds to the binding substance (BS, lipoteichoic acid) on the recipient cell to give the mating aggregates so characteristic of conjugation. Interestingly, both AS and BS have been associated with increased virulence in a rabbit endocarditis model with AS having RGD motifs (arginine-glycine-aspartic acid), which are known to promote binding to the integrin family of cell surface proteins.

Once the pCF10 transferosome is established in the transconjugant, a cytoplasmic membrane protein binds pheromone and prevents its release. The inhibitory peptide, iCF10, is synthesized from a shorter version of Q_L called Q_S. A surface exclusion protein, Sea1 or Sec10 (for pAD1 or pCF10, respectively), is also expressed to reduce aggregation between donor cells and provides a second level of control to prevent redundant mating between plasmid-bearing cells.

There also seems to be a close relationship between replication and transfer in these plasmids with the replication protein PrgW embedded within a region that negatively regulates transfer in pCF10. The replication protein also appears to have a requirement for pheromone that is not yet understood. There are two *oriT*s for pAD1: one within the *rep* gene for plasmid replication and a second dominant one near the genes for relaxase (TraX) and the coupling protein (TraW) on the opposite side of the plasmid. The *oriT* of pCF10 is located near *pcfG*, encoding the relaxase, which is interrupted by a functional group II iteron suggesting tight control of the conjugative process.

Streptomyces

The transfer systems found on conjugative plasmids in the large genus *Streptomyces* differ significantly from those of both nonfilamentous, Gram-positive bacteria and Gram-negative systems in that there is only a single essential transfer protein and no evidence for a relaxase or *nic* site has been found. *Streptomyces* are a medically important source of antibiotics and other therapeutic compounds and are thought to be a major reservoir for antibiotic resistance mechanisms that protect these bacteria from the arsenal of antibiotics they produce.

The conjugative plasmids of *Streptomyces* range in size and structure from the circular 9 kb plasmid pIJ101 to the large linear 350 kb plasmid SCP1. The phenomenon of conjugation in this genus was first identified by the ability of certain integrating plasmids to mobilize chromosomes (Cma). One such plasmid SLP1 (17 kb) excises and integrates at the 3′ end of an essential tRNA^{Tyr} gene in *Streptomyces lividans* with tRNA genes providing the loci for integration of a number of phage and plasmids.

Streptomycetes are soil microorganisms that undergo a complex differentiation program whereby spores germinate and form substrate mycelia that penetrate into the support surface followed by the erection of aerial hyphae that are multinucleate mycelia. These mycelia eventually septate and form spores. As the cells enter the hyphal stage, they begin to produce an array of secondary metabolites characteristic of the organism and also enter a phase where they are competent for conjugation between aerial mycelia or mycelia and other organisms such as *E. coli*.

The intermycelial transfer of a plasmid requires one essential protein, for example, Tra, in pIJ101, which has homology to the proteins FtsK and SpoIIIE. This protein is located at the asymmetric septum of sporulating *B. subtilis* cells and ensures that a copy of the chromosome enters the forespore. Since no relaxase protein has been found associated with pIJ101, it appears that this might be a conjugation system closely related to partitioning mechanisms involving double-stranded DNA.

Once the DNA has been transferred to one compartment of a long mycelium, the plasmid is distributed to all compartments by the *tra* and the *spd* gene products. This process slows the growth rate of the cell and areas on agar plates where plasmids are spread via inter- and intramycelial transfer form ‘pocks’ of more slowly growing cells, which resemble plaques on a phage titer plate. This phenomenon has proven useful in identifying cells containing conjugative plasmids.

Mobilization

Mobilization is a widespread phenomenon whereby a smaller plasmid encoding its own *nic* site and Dtr genes (*mob*) utilizes the transport machinery of a usually larger plasmid to effect its own transfer. Many plasmids can be mobilized by plasmids from a number of Inc groups. For instance, ColE1 can be mobilized by plasmids from IncF, -P and, -I groups and less effectively and with different requirements by IncW plasmids. The Mob proteins of ColE1 consist of MbeA (relaxase) and MbeB and C that aid in relaxosome formation. MbeD has an Eex function. In the vector pBR322, derived from ColE1, the *mob* genes are deleted and only an *oriT* region for IncP plasmid mobilization remains. While ColE1 requires TraD, the coupling protein, from F for mobilization, the closely related plasmid CloDF13 supplies its own TraD-like protein, a difference that is commonly seen among mobilizable plasmids.

The most remarkable mobilizable plasmid is RSF1010 and its relatives (~8.6 kb in size) from the IncQ group. These plasmids are mobilized very efficiently by plasmids from the IncP group into an extremely broad group of recipients including bacteria, yeast, and plants. This plasmid encodes three Mob proteins, with MobA being the relaxase. Like ColE1, it requires TraG of RP4, a coupling protein, for efficient conjugation. The *oriT* region is a mere 38 bp in size and is homologous to *oriT* regions in plasmids from Gram-positive bacteria, all of which use a rolling circle mechanism during transfer. RSF1010 can be mobilized into plants and between agrobacteria by the *vir* region (not *tra*; see ‘Transfer to plants’) and between strains of *Legionella* using the virulence determinants encoded by the *dot* and *icm* loci involved in macrophage killing (see ‘Evolutionary relationships’).

Small Gram-positive plasmids are also mobilizable by self-transmissible plasmids but less is known about them although recent studies confirm that they encode T4SS homologues and require a transglycosylase (SLT) (**Table 2**). The utility of conjugative transposons as genetic tools in Gram-positive bacteria has overshadowed interest in these plasmids that tend to be mobilized at low frequencies over long periods. One plasmid that replicates and transfers via the rolling circle mechanism using different origins is pMV158. It encodes a relaxase (MobM) that cleaves at a *nic* sequence unique to a group of mobilizable plasmids found in Gram-positive bacteria representing the fourth class of *nic* sequences.

Transfer to Plants

The phenomenon of DNA transfer from *A. tumefaciens* to plant cells has features of both Gram-negative (pilus expression) and Gram-positive (induction of *tra* gene expression) bacteria and has been dealt with separately (**Figure 3**). *A. tumefaciens* carrying large conjugative plasmids such as Ti (tumor-inducing) or Ri (root-inducing) greater than 200 kb cause crown gall disease in plants whereby they induce the formation of tumors at the site of infection. The Ti plasmid encodes a sensor-response regulator system, VirA and VirG, which in conjunction with ChvE, a chromosomally encoded periplasmic sugar-binding protein, process signals from wounded plant tissue. The phosphotransfer reaction from VirA to VirG induces gene expression from the *virA*, *-B*, *-D*, *-E*, and *-G* operons on the Ti plasmid. In addition, the *virC*, *-F*, and *-H* operons are induced but these operons express inessential gene products that affect host range or the degree of virulence. The signals generated by the plant include phenolic compounds, simple sugars, and decreased pH or phosphate content among others.

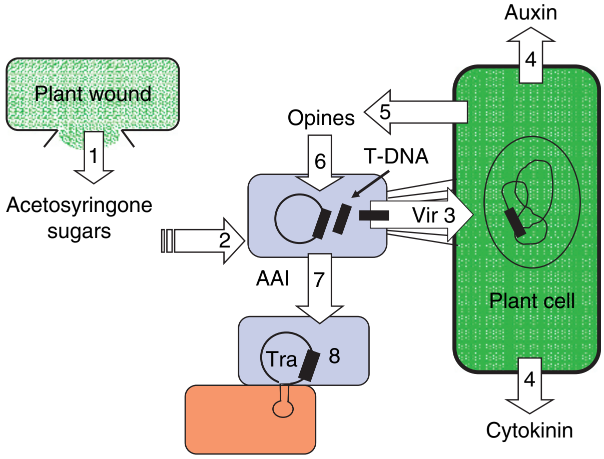

Figure 3 Signaling pathway used to stimulate T-DNA complex transfer to the plant nucleus and Ti conjugative transfer between Agrobacteria. Wounded plant tissue releases phenolics (acetosyringone) and sugars (1) that are detected by the two-component VirA and VirG regulatory system (2). This induces expression of the *vir* genes that encode the transfer apparatus at the pole of the cell that transports the T-DNA to the plant nucleus (3). The T-DNA is incorporated into the plant genome and produces the phytohormones auxin (indoleacetic acid) and cytokinin (4) that trigger tumorigenic growth of the plant tissue. The plant also produces opines (5) whose synthesis is encoded on the T-DNA. These unusual amino acids serve as a food source for *Agrobacterium* and also result in the induction of synthesis (6) of the conjugation factor, *N*-β-oxo-octanoyl-homoserine lactone (AAI; 7). AAI allows quorum sensing, which determines cell density with respect to Ti-plasmid-bearing cells resulting in conjugative transfer to other agrobacteria (8).

The *virB* region encodes 11 proteins that are homologous to the gene products in the Tra2 region of RP4 and are distantly related to the gene products of F. They encode the gene for prepropilin (VirB2), which is processed to pilin via a mechanism similar to that for RP4 pilin. A potential peptidase, homologous to TraF in RP4, has been identified (VirF) but its role has not been proven. The assembly of the VirB pilus is highly temperature dependent with an optimum of 19 °C, which is also maximal for the transfer process. The pili along with the T4SS transfer apparatus are localized to the pole of the cell, where transfer occurs.

The specific segment of single-stranded DNA that is transferred to the plant nucleus is called the T-DNA and can be characterized by the right (RB) and left (LB) borders, which are direct repeats of 25 bp. The T-DNA of nopaline-producing Ti plasmids is about 23 kb in length and contains genes for plant hormone expression (13 kb), a central region of unknown function, and a third region for opine (nopaline) biosynthesis (∼7 kb). The relaxase, VirD2, in conjunction with VirD1 that is similar to RP4 TraJ, cleaves at RB and subsequently LB in a TraI-like manner and remains attached to the 5′ end. VirC1, which binds to an 'overdrive' sequence near the RB, and VirC2 of certain Ti plasmids, enhance T-intermediate formation. Unlike other transfer systems, many copies of the T-DNA segment accumulate in the cytoplasm suggesting replacement replication is important in this system. The accumulation of T-DNA strands has been puzzling but might represent a strategy by the bacterium to ensure infection of the larger, more complex plant cell.

The DNA in the VirD2–T-DNA complex (T-complex) is thought to be coated with the single-stranded DNA-binding protein, VirE2, in preparation for transport through a conjugation pore composed of the VirB proteins (VirB2–VirB11). The VirD4 coupling protein (an ATPase) appears to work in conjunction with VirB11, another ATPase specific to P-like T4SS systems, to transport the T-complex (**Figure 2(b)**). Recent evidence suggests that VirE2 and VirD2–T-DNA transport are uncoupled and can occur using separate transfer pores. One of the Vir proteins, VirB1, which is inessential, resembles the transglycosylase of F (Orf169) and RP4 (TrbN) whereas a truncated version of VirB1 (VirB1*) is excreted into the rhizosphere and mediates adhesion between the bacterium at the site of transfer and the plant. Once the T-complex has entered the plant cytoplasm, the DNA is transported to the nucleus via nuclear localization signals (NLS) on the VirE2 and VirD2 proteins. The T-DNA is randomly integrated into the plant genome whereupon it begins to elicit signals for plant hormone production resulting in tumor formation. The T-DNA encodes for the synthesis of auxin (indoleacetic acid) and cytokinin isopentenyl adenosine, plant hormones that elicit uncontrolled growth at the site of infection. The bacteria derive nutrients from the tumor by the devious method of opine (unusual amino acid) production encoded by the T-DNA. Opines can be classified into about nine different types of compounds including octopine, nopaline, and agrocinopine, with up to three different opines being encoded by a particular T-DNA. Thus Ti plasmids are often referred to as octopine- or nopaline-type plasmids, for instance, depending on the opine they specify. The opines are excreted from the plant and taken up by the *A. tumefaciens* bacteria encoding a region on the Ti plasmid involved in opine utilization. The genes for opine catabolism (e.g., Occ for octopine catabolism) match the genes for the synthesis of that class of opines on the T-DNA.

An interesting aspect of Ti plasmid biology is the induction of conjugative transfer between agrobacterial cells in response to the presence of opines. The genes for this process (*tra*) are distinct from the genes for T-DNA transfer (*vir*) and encode a transfer region with homology to RP4 as well as the *vir* region itself.

Conjugative transfer by Ti has a narrow host range, limited to the genus of *Agrobacterium.* However, the host range can be extended to *E. coli* if an appropriate replicon is supplied, suggesting that it is plasmid maintenance and not conjugative functions that affect host range.

The process of inducing conjugative transfer in these bacteria is unique and fascinating. Initially, there is a low-level uptake of opines, which activates the regulatory protein OccR in octopine-type plasmids and inactivates the repressor protein AccR in nopaline-type plasmids such as pTiC58. This leads to increased expression of the *tra* and opine utilization genes by activation of TraR. TraI (not to be confused with relaxase proteins of F and RP4 plasmids) is a LuxI homologue that synthesizes a signaling compound, *N*-β-oxo-octanoyl-homoserine lactone (*Agrobacterium* autoinducer or AAI), belonging to a diverse class of homoserine lactone-like (HSL) compounds involved in quorum sensing or gene activation in response to changes in cell density (**Figure 3**). TraR is a LuxR-like regulatory protein that detects increased levels of AAI and induces transfer gene expression to maximal levels. The result is the dissemination of the genes for opine utilization among the agrobacteria in the rhizosphere. Thus the system demonstrates a certain degree of chauvinistic behavior since the original colonizer of the plant cell shares its good fortune with its neighbors who then outcompete other bacteria in the rhizosphere.

Evolutionary Relationships

With the advent of high-throughput sequencing and easily available databases, comparison of gene sequences has become routine. Considering that the mechanism for conjugation varies surprisingly little among the systems described above, the high degree of relatedness of these systems with one another is expected (**Table 2**). However, the remarkable finding that there is homology between conjugative systems and the transport mechanisms for a number of toxins and virulence determinants has generated increased interest in these systems. There is almost gene-for-gene homology between the transport system for pertussis toxin of *Bordetella pertussis* (*ptl*) and the *virB* region of the Ti plasmid, which is, in turn, homologous to the genes for pilus synthesis in IncN, -P, and -W transfer systems and is distantly related to those of F-like plasmids. More recently, five *vir* homologues (VirB4, -9, -10, -11, and VirD4) have been found in the *cag* pathogenicity island of *Helicobacter pylori* and some of the Dot/Icm proteins involved in the pathogenesis of *Legionella pneumophila*, which can mobilize RSF1010, are homologous to genes in the Tra2 region of RP4 as well as the Trb region of the IncI1 plasmid R64. In fact, many bacteria carry more than one T4SS that are involved in conjugation, transformation, DNA release, and protein translocation. The number of orthologues is astounding and the versatility of T4SS is only now being appreciated.

Conjugation in Natural Environments

While the process of conjugation is thought to be relevant to the adaptation of organisms to environmental conditions such as the acquisition of antibiotic resistance under continuous pressure for selection, there is much to be learned about the process in nature. Conjugation can be demonstrated in a wide number of situations including the gut of animals, biofilms, soil, aquatic environments including wastewater, and on the surface of plants and animals. However, the level of transfer is usually very low. Most experiments have utilized common lab strains and plasmids, which are good model systems for study but might be irrelevant in nature. Considering the diversity of bacterial species, their vast numbers, and the timescale for their evolution, we have only scratched the surface of this phenomenon in the natural environment. However, studies on domesticated lab strains and plasmids have allowed predictions about the conditions that favor transfer. Most conjugative systems require actively growing cells in exponential phase and have a fairly precise temperature optimum. The majority of systems studied to date mate more efficiently on solid media. Those systems that mate efficiently in liquid media seem to be found in enteric bacteria and might be associated with diseases transmitted via water. More information is required on the natural hosts for conjugative elements and their contribution to the evolution of these elements in an ecological niche. In addition, the most likely route of transmission, which appears to involve many intermediate organisms, is usually impossible to predict or detect because of the complexity of the system and the unknown role of nonculturable organisms in this process. Thus, we can isolate a plasmid from its environment and we can find evidence for its transfer to a new species but we cannot, at this time, follow the plasmid as it makes its way in the world.

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Continuous Cultures (Chemostats)

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Glossary

auxostat A continuous culture system in which the rate of feeding is determined by controlling a single growth-dependent parameter while all other parameters, including the dilution rate (*D*) and the specific growth rate (μ), are adapted accordingly.

chemostat Named from the phrase **chem**ically **stat**ic, the chemostat is a continuous culture system in which the dilution rate (*D*), and hence the specific growth rate (μ), is set externally and all other growth parameters will adapt accordingly.

continuous culture An 'open' culture system in which fresh (sterilized) medium is introduced at a steady flow rate (ϕ), from which the culture fluid is continuously removed at the same rate.

critical dilution rate (D_C) Rate at which $D = \mu_{max}$ in which cells are washed-out faster than they can grow.

C_s Concentration of growth-limiting substrate (*S*) in fermentor (mol l^{-1} or g l^{-1}).

$\bar{C}_s$ Steady-state concentration of growth-limiting substrate (*S*) in fermentor (mol l^{-1} or g l^{-1}.)

C_{Si} Concentration of substrate in medium supply (g l^{-1}).

C_X Concentration of biomass in fermentor (g DW l^{-1}; DW = dry weight of cells).

$\bar{C}_X$ Steady-state concentration of biomass in fermentor (g DW l^{-1}).

dilution rate (D) Flow rate of incoming medium divided by the volume of the culture in the continuous culture vessel (h^{-1}).

fermentor Cultivation vessel with appropriate stirring device and control of temperature, aeration, gas supply, pH, pO_2, and ports for addition and removal of gas and liquids.

flow rate (ϕ) Rate at which fresh medium is supplied to the fermentor (l h^{-1}).

maintenance coefficient (q_m) Maintenance energy requirement of the biomass in culture (g or moles substrate (g DW h)$^{-1}$).

maximum specific growth rate (μ_{max}) Rate of increase in biomass (h^{-1}) relative to biomass present when all nutrients are present in excess and no growth inhibitors are present.

Monod saturation constant (K_S) Substrate concentration (g^{-1} or mol l^{-1}) at which the specific growth rate is equal to half of the maximum specific growth rate.

specific consumption rate (q_s) Substrate (g or moles) consumed per gram biomass per hour, also equal to μ/Y.

specific growth rate (μ_s) Rate of increase of biomass relative to the biomass already present (h^{-1}), numerically equal to $\ln 2/t_d$. The subscript 's' refers to the growth-limiting substrate, but is used optionally.

steady state Condition of a continuous culture in which changes in cell density and physiological state of the cells are no longer detectable.

turbidostat A continuous culture with a growth-dependent feedback system, in which the dilution rate is controlled by an internal sensor monitoring turbidity.

Y''_{SX} (yield factor for biomass on substrate) Quantity of cells produced per substrate consumed (g DW produced per g or moles substrate consumed).

Y^{max}_{SX} True yield factor for biomass on substrate, that is, the experimental yield factor Y''_{SX} if no maintenance exists (g DW produced per g or moles substrate consumed).

Abbreviations

GOGAT-GS	glutamate oxoglutarate amino transferase and glutamine synthetase
PMF	proton motive force
PTS	phosphotransferase system

Defining Statement

Continuous culture techniques enable the cultivation of microorganisms at submaximal growth rates under highly controlled, nutrient-limited conditions and, if desired, at high cell density, thereby providing researchers with powerful and reproducible tools that have several advantages over batch cultivation. Continuous cultivation is invaluable for (eco)physiological investigations of microorganisms, including functional genomics studies.

Introduction

Continuous culture is a set of techniques used to reproducibly cultivate microorganisms at submaximal growth rates at different growth limitations in such a way that the culture conditions remain virtually constant (in 'steady state') over extended periods of time. In the steady state, the growth of organisms can be studied in great detail under precisely controlled physiochemical states. Such conditions are amenable to a great deal of mathematical modeling that enables powerful quantitative analysis of microbial activities. Continuous culture principles first appeared in the literature near the middle of the twentieth century, notably from work performed in the labs of Herbert, Monod, and Novick. Since that time, continuous culture techniques have become common tools in both research and industry. A large diversity of continuous culture applications exists, of which only a modest subset will be mentioned in the present work. Focus will be on a number of classic and a few up-to-date examples of the use of continuous cultivation in various applications. As will be described, the use of continuous culture has enabled studies into several ecological phenomena, including the relationship between growth rate and intracellular metabolic fluxes, the transcriptional responses of microorganisms to various nutrient limitations, the competitive strategies between microorganisms at low nutrient concentrations, as well as the selection and competition between spontaneous or designed mutants for biotechnological applications. As synergistic tools continue becoming more powerful and widely available, the number of uses and the value of the classic continuous culture techniques will likely continue growing at a comparable rate.

Theory of Continuous Culture

General

Microorganisms, inoculated into a suitable growth medium, will grow at a rate that is the maximum possible under the given conditions. During their growth, the environment will continuously change, but as long as the conditions remain favorable, growth will continue until at least one of the essential substrates in the medium becomes limiting. If all other nutrients are present in excess, this is called the growth-limiting substrate. The specific growth rate of a microorganism is dependent on the concentration of the growth-limiting substrate according to the empirical equation of Monod (1942, 1949):

$$\mu = \mu_{\max} \frac{C_s}{K_s + C_s} \tag{1}$$

where μ is the specific growth rate, $\mu_{\max}$ the maximum specific growth rate, C_s the concentration of the growth-limiting substrate, and K_s the Monod saturation constant, which is numerically equal to the substrate concentration at which $\mu = \mu_{\max}$. We will use this very simple mathematical model for growth to introduce the mathematics of the operation of the chemostat. Note that the doubling time (t_d) is related to μ as $t_d = \ln 2/\mu$.

To obtain sizeable population densities, the substrate concentrations employed in a batch culture are much higher than K_s (K_s values are usually in the nmol l^{-1} range), so that growth occurs at the maximum specific growth rate. Most microbial and biochemical research has been carried out on microorganisms grown under these conditions. An experimental drawback of the batch culture is that during growth the medium composition will change, excretion products may accumulate, and cell density will increase. In view of the adaptability of the physiology of the microbes, this means that the results obtained from such cultures will depend on the time of harvest. Another drawback of cultivation in batch is that questions concerning general physiology, such as cellular metabolic pathways, composition, and enzymology at submaximal growth rates under nutrient-limited conditions, cannot be answered.

In contrast, in continuous culture, it is possible to maintain steady-state concentrations of a growth-limiting nutrient in the culture, which permits growth of microorganisms at submaximal rates. In addition, in continuous culture, parameters such as pH, oxygen tension, concentration of excretion products, and population densities can easily be controlled. Several types of continuous culture methods exist (i.e., auxostat, turbidostat, and the chemostat), but by far the most common is the flow-controlled continuous culture, the chemostat, which will be discussed first.

As already mentioned, chemostat is used for the cultivation of microorganisms at growth-limiting substrate concentrations. In the medium reservoir all the compounds necessary for growth are present in excess, with the exception of the growth-limiting substrate, C_S. Under these conditions, the microorganisms grow at a specific growth rate, μ, which is lower than the maximum specific growth rate, μ_{max}. The chemostat is an 'open' culture system (often a lab-scale growth vessel or fermentor) in which fresh (sterilized) medium from a medium reservoir is introduced at a steady flow rate, ϕ, from which the culture fluid emerges at the same rate, often by a simple overflow system. With a constant volume, V, and an inflow rate, ϕ, the dilution rate, D, is defined as

$$D = \frac{\phi}{V} \qquad (2)$$

where the dilution rate is expressed in h^{-1}.

Monod showed that over a large range of growth rates, a fixed relationship exists between the amount of substrate consumed and the amount of biomass produced:

$$\frac{dC_X}{dt} = -Y''_{SX}\frac{dC_S}{dt} \qquad (3)$$

where dC_X/dt is the change in biomass concentration over time. Y''_{SX} is the yield factor and is defined as the amount of cell material produced per amount of substrate consumed. It should be remembered that Y''_{SX} is only a constant in the simple mathematical model of Monod, as will be seen later.

Biomass: If the medium in the fermentor is inoculated, for example with bacteria, the culture will grow at a given rate. At the same time, a quantity of bacteria will be washed out via the overflow, because the culture is continuously fed and diluted with fresh medium from the medium supply. For the culture it thus follows that the accumulation of biomass is equal to growth minus washout or

$$\frac{dC_X}{dt} = \mu C_X - DC_X = (\mu - D)C_X \qquad (4)$$

Hence if $\mu > D$, C_X will increase, while if $\mu <$ D, C_X will decrease. If $\mu = D$, an equilibrium will exist. While the formula given above accurately describes the general situation, it can easily be shown that, starting from non-steady-state conditions, a steady state must inevitably be reached, provided that D does not exceed the critical value D_C:

$$D_C = \mu_{max}\frac{C_{Si}}{(K_S + C_{Si})} \qquad (5)$$

where C_{Si} is the concentration of the growth-limiting substrate in the medium supply. If $C_{Si} >> K_S$, which is usually the case, then $D_C \approx \mu_{max}$. However, if $C_{Si} << K_S$, the culture will be washed-out at $\mu << \mu_{max}$. If $D < D_C$, the establishment of a steady state may be considered as follows. At $\mu > D$, the biomass concentration, C_X, will increase. Owing to the resulting decrease in substrate concentration (C_S), the specific growth rate μ will then decrease. If μ becomes lower than the dilution rate, D, then C_X will decrease because of washout. Consequently, C_S will increase again. Therefore, it can be concluded that the dynamic equilibrium $\mu = D$ is a stable situation. Accordingly, a steady state will be established automatically. It is usually assumed that a steady state has been reached if C_X has not changed during two volume changes and at least five volume changes, in total, have occurred.

Growth-limiting substrate: Substrate enters the culture vessel at a concentration C_{Si}. Consumption of the substrate by the organisms results in a concentration C_S. The net rate of change in the culture vessel is obtained by a balance equation:

$$\frac{dC_S}{dt} = \text{in} - \text{out} - \text{consumption} \qquad (6)$$

in which

$$\text{consumption} = \frac{\text{growth}}{\text{yield}} = \frac{\mu C_X}{Y''_{SX}}$$

It follows that

$$\frac{dC_S}{dt} = D(C_{Si} - C_S) - \frac{\mu C_X}{Y''_{SX}} \qquad (7)$$

At steady state, dC_X/dt and dC_S/dt are both equal to zero. This, when combined with eqns [4] and [7], gives the equilibrium concentrations $\bar{C}_X$ and $\bar{C}_s$.

If

$$\frac{dC_X}{dt} = (\mu - D)C_X = 0$$

then

$$\mu = D \qquad (8)$$

Hence

$$D = \mu_{max}\frac{\bar{C}_S}{(K_S + \bar{C}_S)} \qquad (9)$$

Or:

$$\bar{C}_S = K_S\frac{D}{\mu_{max} - D} \qquad (10)$$

Furthermore

$$D(C_{Si} - \bar{C}_S) = \frac{\mu \bar{C}_X}{Y''_{SX}} \qquad (11)$$

Combining eqns [11] and [8], it follows that

$$\begin{aligned}\bar{C}_X &= Y''_{SX}\left(C_{Si}-\bar{C}_S\right)\\ &= Y''_{SX}\,\frac{C_{Si}-K_S D}{\mu_{max}-D} \qquad (12)\end{aligned}$$

where K_S, μ_{max}, and Y''_{SX} are constants for a microorganism under the specified condition of temperature, medium composition, and the nature of the growth-limiting substrate, respectively For C_{Si} and D a constant value can be chosen. From eqn [10] it appears that $\bar{C}_s$ solely depends on D. If K_S, μ_{max}, and Y''_{SX} are known for a given microorganism, the relationship between $\bar{C}_X$ or $\bar{C}_s$ and D can be predicted at a chosen C_{Si}. This is illustrated in **Figure 1**.

The amount of cell material produced per unit of time is given by $D\bar{C}_X$ (g l^{-1} h$^{-1)}$. This term is also known as the productivity. Note that the theoretical lines often do not follow experimental values when the dilution rate is below ~10% of μ_{max}. This is because at lower μ, the assumption that Y''_{SX} is a constant (i.e., the Monod model for growth) does not hold (see below). Equation [12] shows that $\bar{C}_X$ depends on D and C_{Si} and is proportional to C_{Si} if $\bar{C}_s << C_{Si}$, which is usually the case in the experimental lab situation. At varying C_{Si}, the relationship between D and $\bar{C}_X$ or $\bar{C}_s$ is illustrated in **Figure 2**.

It is important to note that $\bar{C}_s$ is independent of C_{Si}. At dilution rates well below D_C, relatively high cell concentrations can be obtained at very low, growth-limiting concentrations of the substrate. Hence, high biomass samples of cells that are growing at a submaximal rate and maintained in an active, controlled physiological state, are available for (eco)physiological studies. This is one of the great assets of chemostat cultivation.

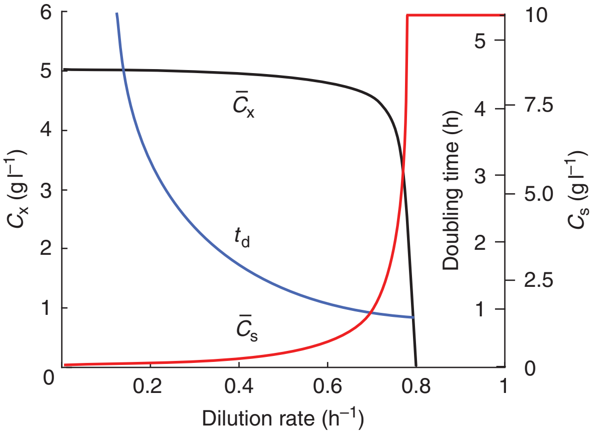

Figure 1 Steady-state relationships in a continuous culture (theoretical). The steady-state values of substrate concentration, bacterial concentration, and doubling time at different dilution rates are calculated from Eqns [10] and [12], for an organism with the following growth constants: $\mu_{max}=0.8\,h^{-1}$, $Y''_{SX}=0.5\,g\,g^{-1}$, $K_S=0.15\,g\,l^{-1}$, and a substrate concentration in the medium supply of $C_{Si}=10\,g\,l^{-1}$.

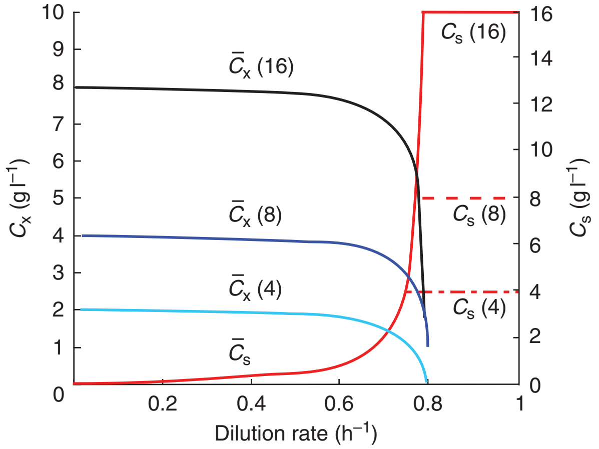

Figure 2 Effect of varying the concentration of substrate in the medium supply (C_{Si}) on the steady-state relationships in a continuous culture (theoretical). The curves are calculated from Eqns [10] and [12] for an organism with $\mu_{max}=0.8\,h^{-1}$, $Y''_{SX}=0.5\,g\,g^{-1}$, and $K_s=0.15\,g\,l^{-1}$, for three media with different substrate concentrations of 4, 8 and 16 g l^{-1}.

In practice, the parameters of a culture such as μ_{max}, Y''_{SX}, and K_S can be determined in various ways. In the chemostat, μ_{max} can usually be determined more reliably than in a batch culture for the following three reasons: (1) prior to the determination of μ_{max} the culture can be grown at a rate close to μ_{max}, ensuring that all cells are optimally adapted to growth at their near-maximum rate; (2) lag phases will not interfere with the measurement; and (3) possible influences of changing the substrate and the product concentrations are minimized. For the actual measurement, the dilution rate is increased (in one step) from a value slightly below μ_{max} to a value of 20–50% above the critical dilution rate. This results at once in alleviation of the substrate limitation and in gradual washout of the culture. The rate at which this washout proceeds can be expressed as given in eqn [4], which after integration gives

$$\ln C_X = (\mu - D)t + \ln C_{Xo} \qquad (13)$$

where C_{Xo} represents the cell density at the start of the washout period. Because the substrate is no longer limiting, the culture grows at μ_{max} and a plot of ln C_X versus t yields a line with a slope of $\mu = D$. Because D is fixed at a known value, μ_{max} can be determined.

K_s can be obtained from continuous culture experiments by selecting D so large that $\bar{C}_s$ can be measured. Usually $\bar{C}_s$ can only be measured over a small range of dilution rates, and hence the data may give inaccurate results. It is essential to minimize residual substrate consumption during the sampling. This can be done (1) by very rapid sampling in a tube with precooled stainless-steel beads, followed by immediate filtering and (2) by decreasing the C_{Si} so that the steady-state biomass concentration, C_X, and hence the rate of consumption, $\mu C_X / Y''_{SX}$, are lowered. A common way to obtain K_S is to perform a nonlinear fit (with least-squares regression), of the measured C_S values in the Monod equation ($\mu = D$) with a proper computer-fitting program. The most accurate method is to use the formula for the specific consumption rate (q_s), discussed below (eqn [16]). An outdated method for the graphical determination of K_S is mentioned here because it is very commonly used to linearize eqn [9], which can be rewritten to produce the so-called 'Lineweaver–Burk plot':

$$\frac{1}{D} = \frac{K_S}{\mu_{\max} \bar{C}_S} + \frac{1}{\mu_{\max}} \tag{14}$$

At varying D values, a graphical plot of the reciprocal of $\bar{C}_s$ measured versus the reciprocal of D gives a straight line with an intercept with the y-axis equal to $1/\mu_{\max}$ and an intercept with the x-axis equal to $-1/K_S$. The slope of the line is $K_s/\mu_{\max}$. Practical values of K_s can lie in a range between 10^{-8} and 10^{-3} mol l^{-1}, but are usually between $10^{-7}$and 10^{-5} mol l^{-1}. However this method gives inaccurate results because those points obtained at the low end of the substrate concentration range (which usually are the most inaccurate) have the greatest effect on the position of the line in such a plot. This graphical method has been replaced by the direct linear plot.

The yield factor, Y''_{SX}, can be obtained from batch experiments using a series of cultures with increasing C_{Si} and further by a graphical plot of C_{Si} versus $\bar{C}_X$. However, this method often neither gives data as reproducible nor dependable as that observed from the chemostat. In continuous cultures, Y''_{SX} can be calculated from eqn [12], according to the simple Monod model for growth, but now we must refine the mathematical treatment of the operation of the chemostat, because in practice it is observed that at low growth rate the yield is strongly influenced by the fact that an organism requires maintenance energy for a number of purposes. The resulting deviation is illustrated in **Figure 3**.

In other words, in eqn [12], Y''_{SX} will not always be a constant. The percentage of the total consumed substrate used for maintenance will increase as μ decreases and, thus, Y''_{SX} will decrease as μ decreases. Marr, and later Pirt, have given an explanation based on the assumption that for its maintenance a cell requires a certain amount of energy per unit of time, independent of the specific growth rate. This means that the total consumption of substrate is equal to the consumption of substrate for maintenance plus consumption of substrate for growth:

$$\frac{\mu C_X}{Y''_{SX}} = q_m \cdot C_X + \frac{\mu C_X}{Y^{\max}_{SX}} \tag{15}$$

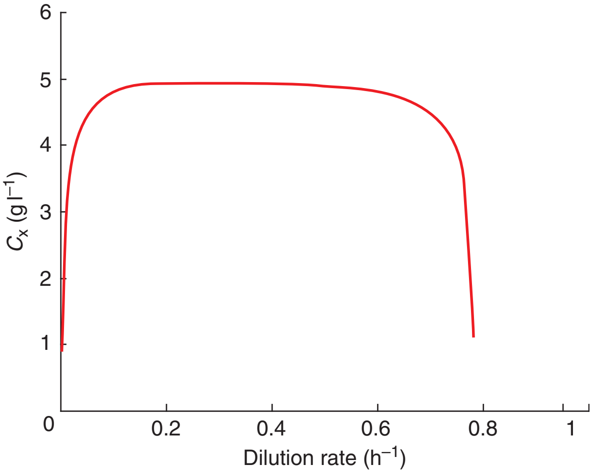

Figure 3 Steady-state biomass concentration $\bar{C}_X$ as a function of the dilution rate D in a chemostat when there is a maintenance requirement for the growth-limiting substrate: $= Y_{SX}{}^{\max} = 0.5$ g g^{-1}, $C_{Si} = 10$ g l^{-1}, $q_S{}^{\max} = 1.6$ g g^{-1} h^{-1}, $q_m = 0.08$ g g^{-1} h^{-1}, and $K_S = 0.15$ g l^{-1}.

If we divide by C_X, we obtain the specific consumption rate of the microorganism, q_s:

$$q_s = \frac{\mu}{Y''_{SX}} = q_m + \frac{\mu}{Y^{\max}_{SX}} \tag{16}$$

where q_m is the specific maintenance energy requirement expressed as amount (moles or grams) of substrate consumed per unit of biomass per unit of time. Note that in the literature we often see the symbol m_s instead of q_m. $Y^{\max}_{SX}$ is the maximum yield (also known as the true yield), that is, the growth yield if no maintenance energy is required. The experimentally obtained $Y^{\max}_{SX}$ value should not be confused with the 'theoretical' maximum yield that can be calculated from metabolic pathways, which is at least twice as high as the $Y^{\max}_{SX}$.

Maintenance energy is necessary, in the first place, to maintain the proton motive force (PMF). This is a proton gradient across the cell membrane that is essential for various metabolic functions (e.g., maintaining ion gradients across the cell membrane). Furthermore, energy is used in the 'turnover' of proteins and mRNA, for repair and motility, among other things. The existence of a maintenance energy requirement can be deduced from the fact that all microorganisms at rest (i.e., not growing) retain a certain respiratory level.

In the older literature, a graphical plot of $1/Y''_{SX}$ versus $1/D$ is used to derive the value of q_m (slope) and $1/Y_{SX}$ (intercept with the y-axis). However, in this type of reciprocal plot the same drawback is observed as pointed out above for the K_S determination. In this case, a plot of q_s against D gives much more dependable results. An example is shown in **Figure 4** (lower line), where the specific rate of oxygen consumption of a carbon- and energy-limited culture is plotted against D. The value of q_s can

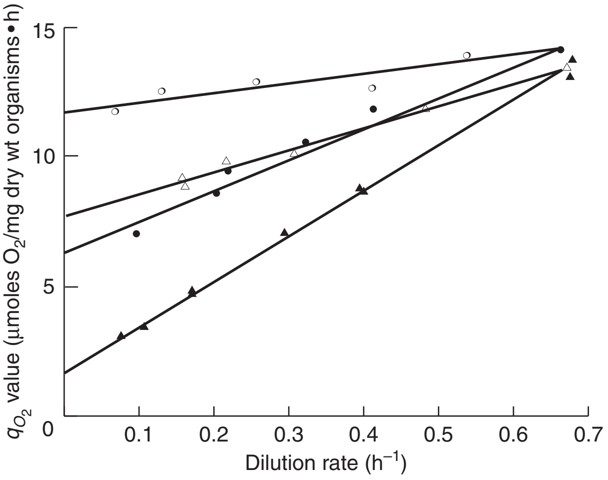

Figure 4 Relationship between the specific growth rate and the specific rate of oxygen consumption in variously limited chemostat cultures of *Klebsiella aerogenes* growing in a glucose-containing medium. Cultures were, respectively, carbon-limited (▲), NH$_3$-limited (△), SO$_4^{2-}$-limited (•), and phosphate-limited (○). Reproduced from Neijssel OM and Tempest DW (1976) Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC-418 in carbon-limited and carbon-sufficient chemostat culture. *Archives of Microbiology* 107: 215–221.

easily be calculated by monitoring the oxygen content of in- and out-flowing gas and by measuring the biomass concentration in the steady state. The specific maintenance coefficient in this case is expressed as $q_{m(O_2)}$, that is, the specific oxygen consumption for glucose respiration at $D = 0$. If the specific glucose consumption rate, $q_{s(glucose)}$, instead of q_{O_2}, would be plotted as a function of D, the experimental yield is measured and the $q_{s(glucose)}$ is calculated from the equation $q_s = \mu / Y''_{SX}$.

Although q_m can directly be read from the graph at $D = 0$, the Y_{SX}^{max} must be calculated from the slope of the curve under carbon and energy limitation. The experimental data indeed often show a linear relationship. In such cases it is assumed that q_m is a constant. However, sometimes a straight line is not obtained. It is clear that in such a case Pirt's concept does not hold. An essential assumption, which was previously not formulated explicitly, is that Pirt's concept can only be applied when growth is energy-limited. This means that under other limitations the 'apparent' q_m may have a variable value (see **Figure 4**), and does not refer to the 'minimum' energy required for maintenance of the integrity of the living cell. In practice, energy limitation often involves growth-limiting amounts of an organic compound that is simultaneously the energy and carbon source. With limitations other than energy (and carbon), much higher consumptions may be observed as glucose may be consumed for purposes other than growth. This is very well described in the literature for microorganisms grown under nitrogen (ammonium) limitation, and is demonstrated in **Figure 5** from an experiment with *Torula (Candida) utilis*, grown under nitrogen limitation. At low growth rates, the cell will store reserve material that does not contain nitrogen (e.g., polyglucose or poly-β-hydroxybutyrate), because the

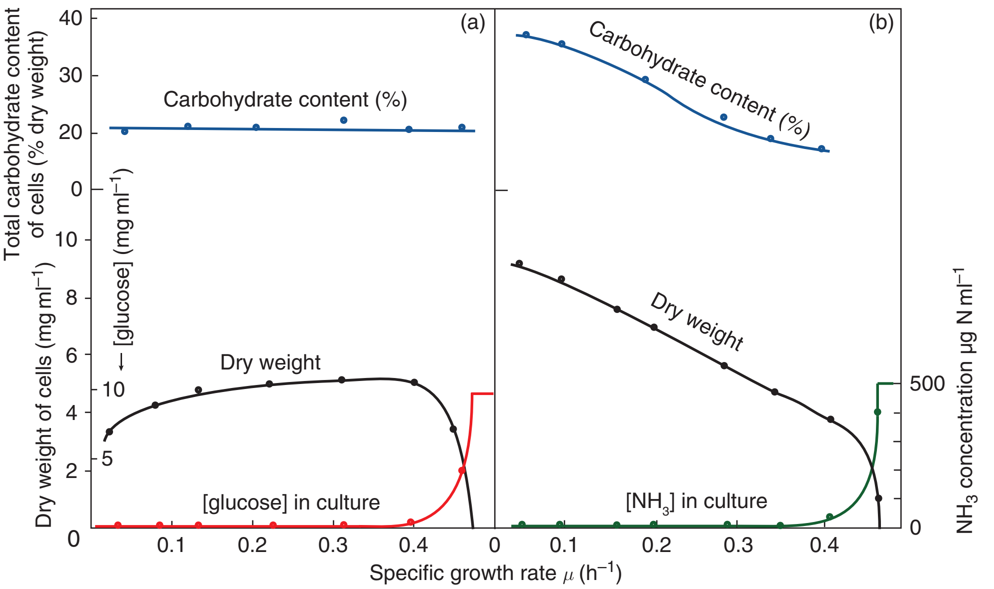

Figure 5 Carbohydrate content of *Torula utilis* as a function of growth rate and limiting nutrient (unpublished data of Herbert and Tempest). The organism was grown in a continuous culture at a number of different growth rates in a glucose-NH$_3$-salts medium (a) with glucose as limiting nutrient, and (b) with NH$_3$ as limiting nutrient. Dry weight of cells in the culture and their total carbohydrate content (anthrone method), as well as steady-state levels of glucose and NH$_3$ in the culture, are plotted against growth rate. Reproduced from Herbert D (1961) The chemical composition of microorganism as a function of their environment. In: Meynell GG and Gooder H (eds.) *Microbial Reaction to Environment*: *11th Symposium*, pp. 391–416. Reading: Society of General Microbiology: Cambridge University Press.

cell has an excess of carbon and energy. This phenomenon is quite common and may have ecological implications, because if a shortage of energy and/or carbon sources occurs, the cell can then use the stored reserve material. This ability of a cell to consume more substrate than strictly necessary for the synthesis of 'standard' cell material is based upon the surplus capacity of the respiratory system under substrate excess. This will be further discussed later.

The consequence of the introduction of the maintenance energy requirement is that the empirical Monod model for growth no longer holds. If maintenance energy is playing a role, then clearly a discrete rate of substrate supply and/or consumption can take place without growth, when the rate of supply is lower than required for maintenance purposes. This implies that the Monod type of saturation curve (hyperbola eqn [1]) must be written for q_s rather than for μ:

$$q_s = q_{\max} \frac{C_s}{K_s + C_s} \tag{17}$$

This is the formula commonly used in computer models to calculate K_S from (weighted) experimental data.

After rearrangements of eqn [17] one obtains an alternative formulation for μ:

$$\mu = \mu_{\max} \frac{C_s}{K_s + C_s} - \frac{K_s}{K_s + C_s} Y_{SX}^{\max} q_m \tag{18}$$

This equation shows that at $C_s = 0$, $\mu = -q_m\ Y_{SX}^{\max}$, while at $C_s = \infty$, $\mu = \mu_{\max}$. This equation is the Monod equation in the first part, corrected with a term that becomes zero at very high C_S. Accounting for maintenance, the formulae for the steady state of C_X and C_S become

$$\bar{C}_X = \frac{[D Y_{SX}^{\max}(C_{Si} - C_S)]}{D + q_m Y_{SX}^{\max}} \tag{19}$$

$$\bar{C}_S = \frac{(D + q_m Y_{SX}^{\max}) K_S}{[Y_{SX}^{\max}(q_s^{\max} - q_m)] - D} \tag{20}$$

Figure 3 has been constructed from eqns [19] and [20], using assumed kinetic parameters: $Y_{SX}^{\max}$, C_{Si} $q_S^{\max}$, q_m, and K_S. Further considerations concerning the maintenance energy requirement will be discussed under the physiological studies.

In continuous culture experiments, many other deviations from the theoretical behavior of chemostat cultures can be found. For example, if the cells present in the culture are not all viable (i.e., if a certain percentage of cells continuously die), a deviation will appear because the living cells must grow faster than D to maintain the value of C_X. Another problem might be the toxicity of the growth-limiting substrate, which could give deviation at high D (i.e., high $\bar{C}_s$). Mathematical models incorporating these extra variables can be found in the literature.

In the laboratory practice there are a number of potential problems related to the growth of microorganisms in the chemostat. Inhomogeneities may often occur because the culture is not well stirred, or because wall growth takes place.

Continuous Cultivation by Other Controls: The Turbidostat and pH-Auxostat

The flow-controlled chemostat is not suited for the growth of microorganisms at a value μ near D_C ($\approx \mu_{\max}$). Small experimental errors in the pumping rate, and hence in the D, will have dramatic effects on $\bar{C}_X$ (see **Figure 1**). In such a case, a turbidostat is preferred. In a turbidostat, the density (turbidity) of the culture is measured continuously and kept constant by a proportional adjustment of the pumping rate. A practical problem is that the measuring device, such as a flow-through cell in a spectrophotometer, used to measure the turbidity and generate the feedback to the pump can easily be fouled by wall growth.

Other types of continuous cultures are controlled by monitoring a variety of measurable chemical or physical parameters and appropriate feed back. Examples are carbon dioxide, sulfide, light, oxygen, and protons (i.e., pH). Such systems are called 'auxostats'. The pH-auxostat has frequently and successfully been used in practice. The theory is as follows. Assuming that during growth protons (H^+) are excreted into the culture liquid, the change in their concentration as a function of time can be expressed as follows:

$$\frac{dH^+}{dt} = \mu C_X h + D[H_R^+] - D[H_C^+] - DB_R \tag{21}$$

where h is the stoichiometry of proton formation per gram dry weight of cells (moles (g DW)$^{-1}$), $[H_R^+]$ the proton concentration in the medium reservoir (moles l^{-1}), $[H_C^+]$ the proton concentration in the culture (moles l^{-1}), and B_R the buffer capacity of the reservoir medium (moles l^{-1}). For the simplest situation, in which only a small difference exists between the pH of the medium and that of the culture, eqn [21] reduces to

$$\frac{dH^+}{dt} = \mu C_X h - DB_R \tag{22}$$

and in steady state with $\mu = D$ the following expression for cell density is the result:

$$\bar{C}_X = \frac{B_R}{h} \tag{23}$$

This shows that the steady-state cell density is a linear function of the buffering capacity of the medium, assuming that h is independent of B_R. Combining eqn [21] with the general nutrient balance for continuous culture eqn [7] yields the following expression for C_S in steady-state cultures:

$$\bar{C}_S = C_{Si} - \frac{B_R}{hY''_{SX}} \qquad (24)$$

Finally, solving the conventional Monod expression (eqn [1]) for the obtained steady-state values of C_S allows a plot of the specific growth rate (μ), the steady-state substrate concentration ($\bar{C}_s$), and the steady-state cell density ($\bar{C}_X$) as a function of the buffering capacity (B_R) (**Figure 6**). As can be seen from this illustration, the specific growth rate of the cells remain at a value close to μ_{max} over a large range of buffering capacities but, of course, will decrease at high buffering capacities due to the decreasing concentration of remaining growth substrate. Obviously, this effect will be most prominent with cells possessing relatively high K_S values for the substrate used. In principle, this will provide the opportunity to choose the buffering capacity such that the substrate concentration becomes strongly growth rate-limiting, thus creating an overlap with the conventional mode of substrate-limited growth.

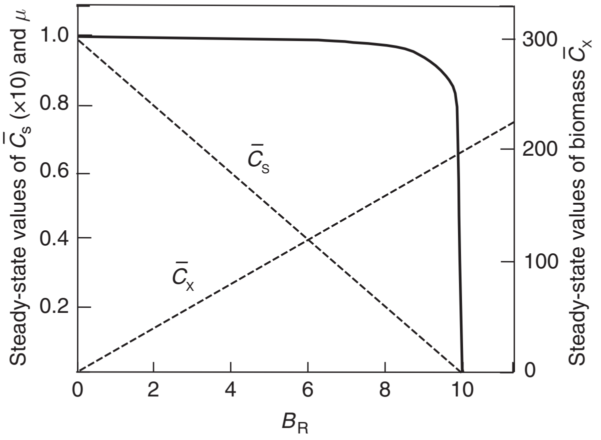

Figure 6 Major growth parameters in a pH-auxostat. Residual substrate concentration C_s (moles l^{-1}), specific growth rate μ (h^{-1}) and cell density C_x (g DW l^{-1}) as a function of the buffering capacity B_R (moles l^{-1}), in the reservoir medium. Arbitrarily chosen values: $Y_{sx}'' = 22$ (g DW (mole)$^{-1}$), h = 0.05 (moles (g DW)$^{-1}$), $C_{si} = 10$ (mole l^{-1}) and $\mu_{max} = 1$ (h^{-1}). Adapted from Gottschall JC (2000) Continuous Culture. In: Lederberg J (ed.) *Encyclopedia of Microbiology*, 2nd edn., vol. 1, pp. 873–886. New York: Academic Press Inc.

Competition of Microorganisms for a Growth-Limiting Substrate

Consider microorganisms A and B, which have μ–C_S curves of the types presented in **Figures 7(a)** and **7(b)**. In the example shown, if organisms A and B are grown in batch culture (with a maximum specific growth rate of μ_{max}), organism A will grow faster than organism B in both cases and therefore becomes dominant (assuming that both have started with equal numbers and neither has a lag phase). In the example shown in **Figure 7(b)**, however, if A and B are grown together in a continuous culture with growth limitation by substrate C_S, then, at low D, organism B will dominate. This can be rationalized by assuming a steady state with organism B at $D = ½\mu^B_{max}$. The steady-state concentration of the growth-limiting substrate would be equal to K_S^B. At that nutrient concentration, organism A will grow at a $\mu < D$, and hence it will be washed out. Conversely, if we would have a steady state of organism A at that same D, organism B would be able to grow faster at the concentration of the growth-limiting nutrient than A, and hence would outcompete (replace) organism A. In other words, in spite of the lower μ_{max} of B shown in **Figure 7(b)**, this organism will grow faster than organism A at low $\bar{C}_s$. In fact, it is the

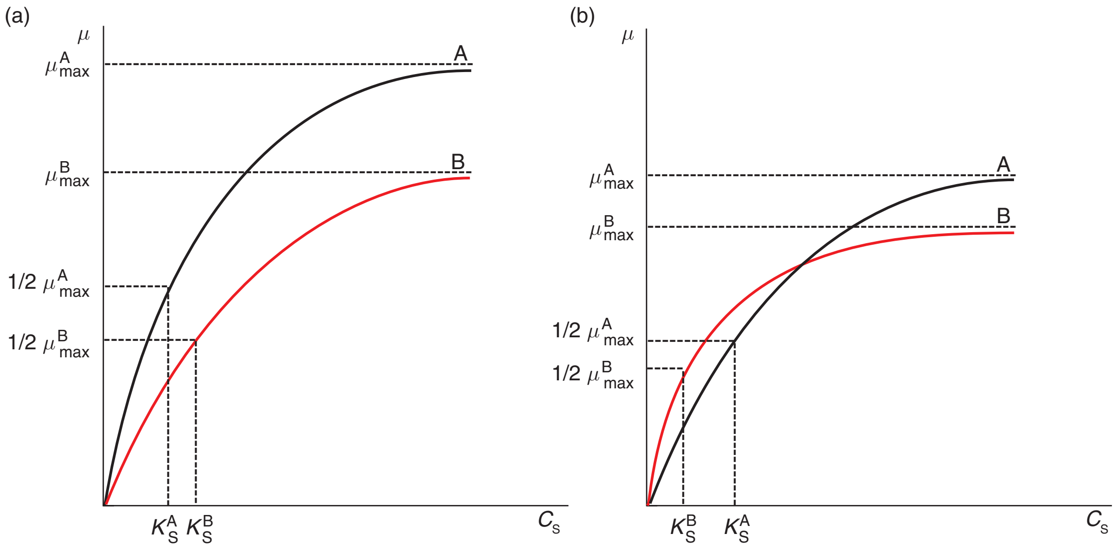

Figure 7 The μ–C_S relationship of two organisms A and B (a) $K_S{}^A < K_S{}^B$ and $\mu_{max}{}^A > \mu_{max}{}^B$; (b) $K_S{}^A > K_S{}^B$ and $\mu^A_{max} > \mu^B_{max}$.

slope of each of the curves, that is $\mu_{\max}/K_S$, which determines the outcome of the competition. This slope is often referred to as the 'affinity' for the substrate. A high affinity, in other words, will equip the organism with the ability to grow relatively fast at very low nutrient concentrations. This is most relevant to the (semi) natural environment. In the later examples of competition for growth-limiting nutrients, we will not only refer to mixtures of different organisms, but also to competition between mutants of one species, which is very important for the general study of selection. However, mutant selection is also a potential practical problem in the continuous cultivation of pure cultures in the laboratory (see below).

Physiological and Functional Genomic Studies with the Chemostat

Physiological Studies

As pointed out in the introduction, the chemostat is a unique tool for the study of the physiology of microorganisms at different growth rates and with different growth limitations under controlled, nutrient-limited conditions. For example, by means of this technique it has been found that the composition of the cell changes strongly, both qualitatively and quantitatively, with changing growth rates and with the type of growth limitation. At low growth rates, cells appear to possess fewer ribosomes (rRNA) than at higher growth rates. This can be directly related to the requirement of the cell to synthesize protein more rapidly when growing fast as the rate of protein synthesis per ribosome is about constant. Especially when growth is limited by the carbon and energy source, the amount of catabolic enzymes (for energy production) present in the cell may be observed to increase with increasing growth rate. Furthermore, the amount of cytochromes in a cell also often increases or decreases in response to environmental changes. However, a general rule for alterations in enzyme activity with changing growth rate cannot be given, because these changes are usually the result of very complex regulatory mechanisms.

Metabolic pathways and growth rates. As already stated, the chemostat is used for the study of all forms of limitation. Examples involving nitrogen and carbon/energy supplies as well as different limitations will be discussed here.

Most organisms grown in batch culture with ammonia as the nitrogen source possess glutamate dehydrogenase for ammonia assimilation. The same enzyme is used by chemostat cultures under limitation by the carbon and energy source. However, if growth is limited by ammonia, the combination of glutamate oxoglutarate amino transferase and glutamine synthetase (the GOGAT-GS system) is induced. The latter enzyme has a considerably lower K_m value for ammonia than does glutamate dehydrogenase (about a factor of 5–10). The assimilation of ammonia through glutamine synthetase costs 1 ATP, but it is clear that under conditions of energy excess, this is not a problem for the cell.

While the biomass yield is, of course, dependent on the substrate (as is the case in batch culture), it is also dependent on the metabolic pathway used by the organism, which in turn may be dependent on the growth rate. The group of Stouthamer has observed a very interesting example. They found that at high growth rates *Lactobacillus casei* ferments glucose, producing lactate. Accordingly, when grown in the chemostat under glucose limitation at high D values, the bacterium produced only lactate. However, when D was lowered, the density of the culture increased and acetate appeared in the culture. The explanation of this phenomenon is that at low D values *L. casei* can make additional ATP by converting pyruvate into acetyl CoA rather than lactate. By means of a phosphoroclastic splitting of acetyl CoA, one additional ATP is formed. To keep a proper redox balance the organism produces formate and ethanol:

$$\begin{aligned}
\text{High } \mu\text{: } & \text{glucose} \rightarrow 2 \text{ pyruvate} + 2\text{ATP} + 2\text{NADH}\\
& \text{pyruvate} + 2\text{NADH} \rightarrow 2 \text{ lactate}\\
& \text{yield: 2ATP}\\
\text{Low } \mu\text{: } & \text{glucose} \rightarrow 2 \text{ pyruvate} + 2\text{ATP} + 2\text{NADH}\\
& 2 \text{ pyruvate} + 2\text{NADH} \rightarrow 2 \text{ formate} + \text{ethanol}\\
& + \text{acetate } + 1\text{ATP}\\
& \text{yield: 3ATP}
\end{aligned}$$

There is no definite answer to the question of why the organism at high growth rates does not also produce energy from glucose as economically as possible. It is possibly due to the fact that the reaction that yields 3 ATP is not fast enough for higher growth rates. It was found that the lactate dehydrogenase is activated by fructose-l,6-biphosphate. At low growth rates the concentration of this intermediate is low and the lactate dehydrogenase does not function. In this situation, the pyruvate can be converted into formate, ethanol, and acetate.

Neijssel and Tempest studied the response of *Klebsiella aerogenes* grown fully aerobically under different limitations in the chemostat with glucose as the only carbon and energy source as shown in **Figure 4**, which is discussed in the theory section. The specific oxygen respiration rate q_{O_2} was recorded as a function of D and in all cases a linear relation existed. In the carbon-limited cultures glucose was completely consumed, but in all other (glucose-sufficient) cultures the sugar was partially respired and also partially fermented, as indicated by the appearance of different products such as gluconate and acetate (not shown in **Figure 4**). In many publications, it is also shown that under limitations other than by the carbon and energy source the cell-composition changed as mentioned for nitrogen limitation. For example, during phosphate or sulfate limitation the cell may synthesize alternative cell wall components not containing the limiting nutrient.

As mentioned earlier, according to the Pirt maintenance concept the extrapolated consumption rate under carbon and energy limitation at $D = 0$ represents the true maintenance energy requirement, q_m. Extrapolation of the other lines to $D = 0$ simply demonstrates that the organism respires more glucose than required for biosynthesis. The apparently wasteful use of glucose under the other limitations may in part be due to the need for more energy for transport of the limiting nutrients, but the authors also clearly showed that a certain uncoupling of energy metabolism and biosynthesis occurred. The interpretation of this phenomenon is that under the other limitations the organism is unable to match (excess) uptake of glucose with the requirement for growth, and hence must waste energy since without it, it would be unable to maintain the proper energy charge and redox balance in the cell. Neijssel and Tempest argue that the maintenance energy requirement is not necessarily limited to the Pirt-type μ-independent maintenance requirement, but may be in part growth rate dependent in a linear manner. The discussion of this point falls beyond the scope of this article.

Yields under fluctuating-nutrient supply. Another point that deserves attention is that energy-limited cultures, when exposed to sudden increases of their limiting energy source, often show lower yields. For example, when glucose-limited cultures of the yeast *Saccharomyces cerevisiae* were given the same amount of glucose per unit of time, but in discrete pulses, rather than continuously, the experimental biomass yield decreased. Directly after the pulse the yeast would excrete ethanol, which would be rapidly consumed again once all glucose had been taken up. The ethanol consumption was proceeding through acetate, which was transiently observed. Before the next pulse of glucose appeared all substrates had been converted into CO_2 or taken up into biomass. Apparently under these conditions, the (excess) uptake of glucose could not be met by biosynthesis, and a certain uncoupling of energy metabolism and biosynthesis would take place and some substrate was wasted as heat. This phenomenon was not observed in the yeast *Candida utilis*, which under these fluctuating conditions would control its glucose uptake to match the requirement for biosynthesis, and hence in the latter case the yields were the same.

Functional Genomic Applications of the Chemostat

With the booming interest in 'functional genomics', which may well be considered as a modern version of microbial physiology, the chemostat has obtained renewed interest not only because it allows one to study the (controlled) response of an organism to different limitations, but also because of the reproducibility of the experiments. This was put to the test by the groups of Pronk and Nielsen, who did a transcriptome analysis of *S. cerevisiae* grown in the chemostat under both aerobic and anaerobic conditions in two independent laboratories. Using triplicate experiments in each laboratory and adequate statistical analysis, they were able to show 95% agreement for transcripts that changed by more than twofold. This is remarkably reproducible for these types of biological experiments and shows the great values of the use of the chemostat for genomic research. The Pronk group also used the chemostat to grow the same organism under carbon, nitrogen, phosphorous, and sulfur limitations at the same dilution rate. In doing so, they could rule out that major differences would be caused by differences in specific growth rate. Experimental design and medium composition were carefully checked to ensure that the chosen growth limitation was realized. They observed that 31% of the annotated genome transcripts changed significantly in these experiments with four separate limitations. Nearly 500 transcripts could specifically be linked to one of the four nutrient limitations. Fifty-one genes showed tenfold changes under one particular limitation. They concluded that the responsible genes may be targets for future diagnostic study and characterization of specific limitations for metabolic engineering strategies. For the cultivation of industrially relevant organisms the use of poorly defined, complex media (such as molasses) is common and hence this type of analysis would be most important. These two examples underline the power and usefulness of controlled cultivation in the chemostat for reproducible and quantitative functional genomic research.

Simultaneous Use of Mixed Substrates in Continuous Culture

Equivalent Substrates

Diauxic growth is a phenomenon that can be observed when, for example, *Escherichia coli* is grown in batch culture on a mixture of glucose and lactose. Growth of the culture clearly shows two phases: first the glucose is consumed and, after a lag phase, the lactose can then be used.

At high glucose concentrations the organism grows at the maximal rate and lactose metabolism is repressed (known as catabolite repression). This is because lactose transport is inhibited at high-energy status of the phosphotransferase (PTS) system. It should be remembered that the PTS system is in dynamic equilibrium with the PMF and the energy charge of the cell. Under simultaneous limitation of growth by glucose and lactose in a continuous culture, at relatively low dilution rate ($D = \mu$), the energy status of the cell is relatively low and the use of lactose does not appear to be repressed. Thus at low *D* values, the simultaneous use of both compounds occurs, whereas at high *D* values lactose does appear in the fermentation broth. In *E. coli*, $\mu_{\max(\text{glucose})}$ is approximately $1.0\,\text{h}^{-1}$, whilst the $\mu_{\max(\text{lactose})}$ is approximately $0.8\,\text{h}^{-1}$. In this case, the complete simultaneous use occurs below $D = 0.5\,\text{h}^{-1}$, and glucose and lactose are detectable only at microgram per liter levels. Between $D = 0.5$ and $0.8\,\text{h}^{-1}$ lactose gradually appears at increasing levels and a plot of C_S (lactose) against *D* shows a normal Monod relation as shown in **Figure 1**. Above $D = 0.8\,\text{h}^{-1}$ lactose is no longer used and the organism shows typical diauxic behavior as in batch culture with excess glucose. The culture was washed out above the D_C for glucose, and hence also for the glucose the organism displayed normal Monod kinetics (**Figure 1**).

The group of Egli has performed similar experiments with other (two or three) mixtures of sugars, for example glucose and galactose, which they were able to measure directly in the steady-state culture liquid at extremely low levels using a new sensitive assay. **Figure 8** shows the results of chemostat experiments run at $D = 0.3\,\text{h}^{-1}$ with different ratios of glucose (0–100%) and galactose (100–0%) in the in-flowing medium.

It can be seen that the residual, steady-state concentration of each of the two growth-limiting substrates was lower in the presence of the second substrate than in its absence. In a 50:50 mixture the residual sugar concentrations were around $20\,\mu\text{g}\,\text{l}^{-1}$ ($\approx 100\,\text{nmol}\,\text{l}^{-1}$), that is, almost half of that compared with the presence of one substrate only. This important observation, predicted by mathematical modeling, indicates that in the competition for substrates in Nature, the capability to use substrates simultaneously has a competitive advantage (also see mixed cultures).

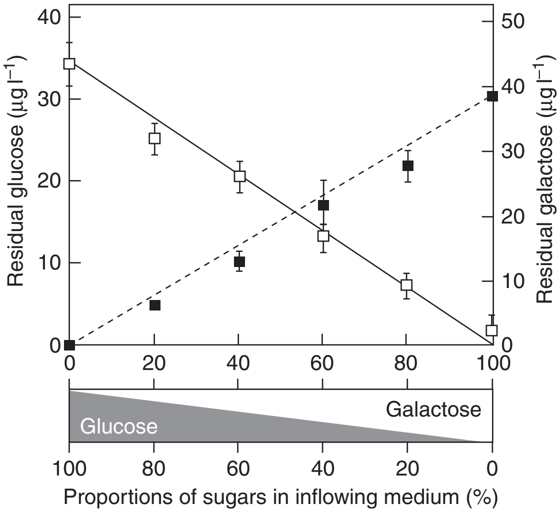

Figure 8 Steady-state concentrations of glucose (☐) and galactose (■) during growth of *E. coli* at a constant dilution rate of $0.30\,\text{h}^{-1}$ in carbon-limited chemostat culture with different mixtures of two sugars. The proportion of the sugars in the mixture fed to the culture is given as weight percentages. The total sugar concentration in the feed was held at $100\,\text{mg}\,\text{l}^{-1}$. Reproduced from Egli T (1995). The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. In: Jones JG (ed.) *Advances in Microbial Ecology,* vol. 14, pp. 305–386. New York: Plenum Press.

The simultaneous, that is mixotrophic, use of substrates with similar roles in metabolism is not limited to sugars but also applies to all kinds of mixtures of carbon and energy sources, mixtures of organic compounds and inorganic energy sources, as well as to mixtures of nitrogen sources, such as ammonium and nitrate. Likewise, the same mixotrophic behavior occurs with respect to electron acceptors such as limiting mixtures of oxygen and nitrate. An extensive study in this area has been done by Gottschal and Kuenen on mixotrophic growth of a facultative sulfur-oxidizing bacterium, *Thiobacillus versutus* (presently named *Paracoccus versutus*) on a mixture of acetate and thiosulfate as an additional energy source and/or CO_2 as an additional carbon source. The organism showed typical diauxic behavior in batch culture at high acetate (consumed first) and thiosulfate, but under simultaneous limitation (at $D = 0.05\,\text{h}^{-1}$) by the two substrates both were used simultaneously. With high acetate to thiosulfate ratio the latter substrate served as supplementary energy source, whilst at low ratio acetate was primarily used as carbon source with CO_2 as supplementary carbon source for autotrophic metabolism. Thus a remarkably efficient metabolic control and use of resources were revealed, which made this organism very competitive under conditions of mixed-substrate supply and/or short-term fluctuation in the supply of the separate substrates.

Simultaneous Limitation by Non-Equivalent Substrates

When in a carbon-limited culture the in-flowing nitrogen (originally in excess) is lowered, the residual nitrogen in the culture will go down. At a stage where, academically speaking, the nitrogen is still in slight excess the organism may, however, begin to induce its high-affinity GS-GOGAT system for ammonium assimilation and hence employ the ATP-requiring assimilation pathway, in spite of its carbon and energy limitation. This was experimentally verified by Egli, who showed that below suboptimal nitrogen content in the in-flowing medium the organism displays an actual physiological double limitation.

A recent study by Ihssen and Egli with glucose-limited *E. coli* cultures confirmed and extended the generally observed phenomenon of de-repression of pathways in the lower *D*-range. Under single limitation by glucose at $D = 0.3\,\text{h}^{-1}$ (i.e., at 40% of $\mu_{\max}$) a large number of metabolic pathways were de-repressed, without the inducer being present, in contrast to what is observed in batch culture. These cultures can metabolize and grow instantaneously on the new substrates, such as sugars, alcohols, and organic acids. It is generally believed that this type of response offers an advantage for survival because it reduces the time required to react to a change in nutrient supply. Clearly under natural conditions, mixed substrates will be available and mixotrophic growth on a variety of substrates will be the rule rather than the exception. In these experiments, the authors ensured that the selection of particular mutants did not play a significant role. This was accomplished in practice by establishing each steady state from a fresh inoculum.

Ecological Studies in the Chemostat

General Considerations

In Nature and in man-made environments such as wastewater-treatment plants, concentrations of nutrients are generally very low. The order of magnitude is usually in the nanomolar (microgram per liter) level and K_S values of organisms are adapted to these low concentrations. The physiological or competitive behavior of organisms at these low levels can be studied by using chemostat cultures grown under the appropriate limitation. It is true that in Nature actual steady states rarely occur but the chemostat is an excellent tool to look at the principles of (eco)physiological response and competition, under controlled conditions. In addition, continuous cultivation in chemostat equipment under dynamic condition can also be done for simulation of natural conditions, though the mathematics of such operations is much more complicated.

The groups of Jannasch and Veldkamp have extensively studied the competition of bacteria under nutrient limitation in the chemostat. It appears that in nature there are many organisms of type B (**Figure 7(b)**), which possess a high affinity for a growth-limiting nutrient (i.e., low K_S combined with a relatively low μ_{max}) for the substrate. An example is that of a rod-shaped bacterium (R) and a spirillum (S) as shown in **Figure 9**.

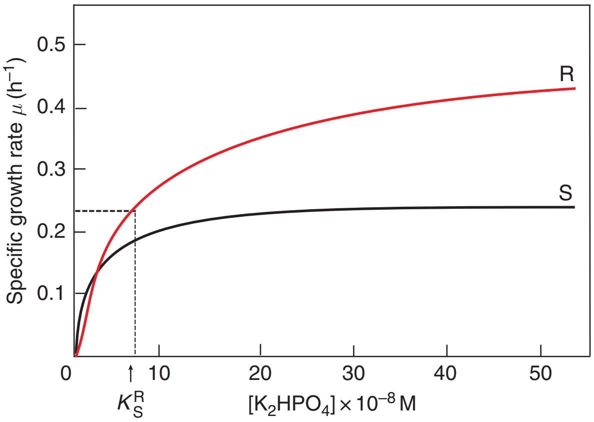

Figure 9 Specific growth rate of fresh water rod-shaped bacterium (R) and a spirillum (S) as a function of phosphate concentration. The curves are schematic and based on two measurements each at the growth rates indicated by the arrows. K_s values: rod-shaped bacterium, 6.6×10^{-8} mol l^{-1}; spirillum, 2.7×10^{-8} mol l^{-1}. Reproduced from Kuenen JG, Boonstra J, Schroder HGJ, and Veldkamp H (1977) Competition for inorganic substrates among chemoorganotrophic and chemooligotrophic bacteria. In: *Microbial Ecology,* vol. 3, pp.119–130.

Both strains were originally isolated from phosphate-limited continuous cultures inoculated with ditch water. One chemostat was run at a high *D*, with a second one at a low *D*. In the culture with the low *D* the spirillum became dominant, whilst in the culture with the high *D* the rod dominated. When the two pure cultures were mixed, and cultivated again at the same *D* values, the cultures appeared to behave like the original chemostat enrichments. When experiments were performed with the same set of two organisms under other nutrient limitations, that is, succinate, ammonium, or potassium, very similar crossing curves were obtained showing that the spirillum had a generally higher affinity for substrates than the rod. This property, which points to a generally higher transport capability at low nutrient concentration, is linked to the much higher surface to volume ratio of the spirillum. This allows the accommodation of more membrane-transport proteins per unit of biomass.

Competition for Mixed Substrates

In nature many growth-limiting substrates are available simultaneously, and hence an understanding of the selection and competition for more than one substrate has also been studied in the chemostat. Unquestionably, other abiotic and biotic variables also play a role in the establishment of a community of organisms in any environment. Theoretical calculations by Fredrickson indicate that the maximum number of coexisting species is determined by the number of variables in a particular environment. As a simple example, a steady state with two growth-limiting substrates will allow a maximum of two existing species in the culture. When a variation of the pH would be admitted as a third variable, the number would increase to a maximum of three.

Gottschal and colleagues published an interesting example of a set of three organisms competing for two substrates. The properties of the three organisms are listed in **Table 1** and refer to *Thiobacillus neapolitanus*, *T. versutus*, strain A2, and *Spirillum* G7.

T. neapolitanus (presently known as *Halothiobacillus neapolitanus*) is a specialized, obligately chemolithoautotrophic sulfur oxidizer, which can grow only on an inorganic sulfur compound (thiosulfate) as the energy source and CO_2 as carbon source. *Spirillum* G7 is a specialized

Table 1 Maximum specific growth rates of two specialists and one versatile bacterium[a]

Organism	*Lifestyle*	*Relevant physiology*	μ_{max} *thiosulfate (T)*	μ_{max} *acetate (A)*	μ_{max} *(T + A)*
Thiobacillus neapolitanus	Specialist	Obligate chemolithoautotroph	0.35	0	0.35
Thiobacillus versutus	Versatile	Facultative	0.1	0.22	0.22
Heterotroph G7	Specialist	Obligate heterotroph	0	0.43	0.43

[a] The specialists can only grow on a single specific substrate. The versatile organism shows diauxic behavior towards thiosulfate, but simultaneously consumes acetate and thiosulfate in the chemostat at low $D = 0.05\,h^{-1}$.

chemoorganoheterotroph, which can grow only on acetate as carbon and energy source. Both specialists have a high μ_{max} in batch (excess substrate) on their respective substrates. In contrast, *T. versutus*, presently known as *P. versutus*, is a facultative organism capable of growing in both modes, but at lower μ_{max} in batch. As mentioned above, when grown in a mixture of the two substrates in batch culture at high substrate concentrations, the organism shows diauxic behavior and consumes acetate first and then thiosulfate. Therefore, μ_{max} (T+A) does not increase. When a mixture of the three organisms is inoculated in excess acetate and thiosulfate in batch culture, we end up with a dominant mixture of the two specialists. However under limitation by a mixture of acetate and thiosulfate at a low dilution rate in the chemostat, the versatile organism can very effectively use the two substrates simultaneously. It was shown that, as a result of this capability at low D ($0.05\,h^{-1}$) it can coexist or even out-compete the two specialists in a mixed culture of the three organisms. This is due the fact that in the mixture of the second substrate (thiosulfate or acetate) the versatile organism can lower the concentration of the other limiting nutrient, as was shown above for the mixture of glucose and galactose (**Figure 8**) and mathematically modeled by Gottschal and Thingstad. In line with theoretical predictions concerning the maximum number of coexisting species in relation to the number of variables, it was shown that *T. versutus* was able to maintain itself at low number in the steady-state culture of *T. neapolitanus*, in spite of its lower affinity for thiosulfate, since the specialist excreted glycollate. The glycollate was mixotrophically consumed by the versatile organism. This demonstrates the important principle that metabolic (excretion) products can not only lead to simple cometabolic consumption of this product by a second population, but that by excreting a consumable product the excreting organism may generate more competition for its main substrate.

Continuous cultivation not only provides a tool to create reproducible steady-state cultures, but also offers controlled alternations of environmental and nutritional conditions, such as feast and famine cycles, changes of substrates, or temperature. In such experiments, a true steady state is not established but nevertheless highly reproducible cycles will allow the precise monitoring of the response of the organism(s). Indeed in the case of the two specialists and the versatile organism, it was shown that the latter could also out-compete the two specialists at low D when acetate and thiosulfate were alternately supplied. As long as the period of the cycle was below 4 h the versatile organism would continue growing, but when longer cycle times were introduced the versatile organism would repress its autotrophic potential too far down to be able to compete with the specialists in the next cycle. This example serves to emphasize the usefulness of continuous cultivation for ecophysiological research.

Industrial Applications of Continuous Culture

In the industrial production of (secondary) metabolites, such as antibiotics, the production organism (i.e., a fungus like a *Penicillium* or a *Streptomyces* sp.) is cultivated at submaximal μ, because only under these conditions will the organism produce at a sufficiently high rate. Clearly for optimization reasons, it is essential to study the performance of the organism in the chemostat at different dilution rates. The production process is, however, rarely performed in continuous culture. The main reasons for this are: (1) the cell density and hence the product concentration can never be very high because the aeration capacity of the fermentors have limited oxygen transfer and cooling capacities. This leads to insufficiently high product concentrations for economical down-stream processing. (2) The inevitable selection of less productive spontaneous mutants under the imposed carbon and energy limitation (see below). Therefore, in industry, the cultivation method of choice is usually fed-batch cultivation. After an initial stage of growth in excess substrate the organism is fed (in batch) with a concentrated feed of the growth-limiting nutrient (i.e., glucose or ammonium) in such a way that the instantaneous consumption of the added nutrient limits the growth of the organism. Using this method, high product concentrations may be reached and in the short-term cultivation period mutants will not become dominant.

In the practice of wastewater treatment many (semi) continuous operations are used, be it that biomass retention and recycling of biomass are practiced. An example of a chemostat-type large-scale process is the production of a 50:50 ammonium/nitrite mixture from ammonium-containing reject water using selected, oxygen-limited mixed cultures of nitrifying bacteria. The effluent is used to feed an anaerobic anammox reactor, which converts the mixture into nitrogen gas as demonstrated by Van Dongen and colleagues.

Competition and Selection of Mutants in the Chemostat

Nutrient-Limited Wild Types and Mutants

A monoclonal bacterial culture will contain mutants. It is known that the average number of mutants for a particular gene is of the order of 1 out of every 10^6–10^9 replications. This means that mutants, which can perform better under nutrient limitation than any wild-type organism, may outcompete the parent strain. Therefore, the study of competition and selection does not require two different species. The technique is sometimes intentionally utilized to isolate mutants, which by spontaneous (or induced) mutation have obtained a competitive advantage. A classical example of this is the development of a constitutive mutant for β-galactosidase of *E. coli* in a lactose-limited culture as described by Novick. Once the constitutive mutant has been enriched, so-called super-constitutive mutants, which produce more β-galactosidase often arise. This is illustrated in **Figures 10** and **11**.

These results can be understood by making the assumption that the uptake of lactose (and the linked conversion of lactose into galactose and glucose) is the rate-limiting step. By producing more permease and β-galactosidase (which is transcribed under the same promoter), the cell would be able to convert lactose somewhat faster at the same $\bar{C}_S$. For example, as long as the lactose permease is the real bottleneck in the rate of metabolism of lactose, a doubling of the permease concentration will allow the organism to maintain the same overall rate at half the concentration of the lactose. Consequently, it will be able to grow at the same rate at half the lactose concentration and hence out-compete the wild type. Consequently, the μ–C_S curve of the mutants changes. This is illustrated in **Figure 11**, where Novick has given the wild-type μ–C_S curve an 'S'-shape to accommodate the fact that, in contrast to the constitutive mutant, it requires a relatively high concentration of lactose to fully initiate transcription of the lactose operon.

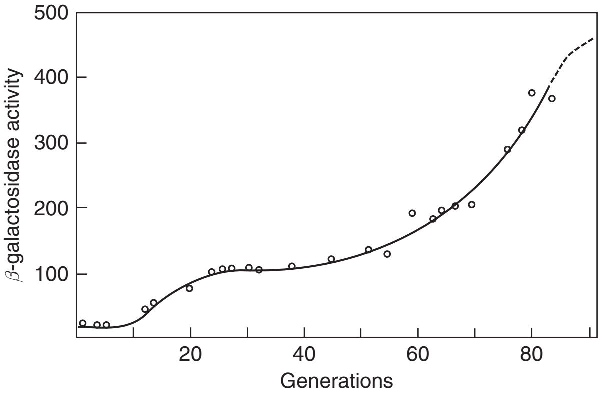

Figure 10 Increase in β-galactosidase activity of *Escherichia coli* strain E-102 grown in a lactose-limited chemostat. Reproduced from Novick A (1961) Bacteria with high levels of specific enzymes. In: Zarrow MX (ed.) *Growth in Living Systems*, *Purdue Growth Symposium*, pp. 93–106. New York: Basic Books Inc.

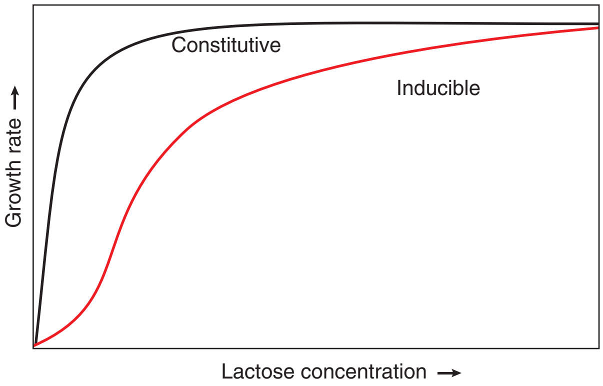

Figure 11 Hypothetical relationship between growth rate and lactose concentration when the growth-limiting enzyme is constitutive and when it is inducible. Reproduced from Novick A (1961) Bacteria with high levels of specific enzymes. In: Zarrow MX (ed.) *Growth in Living Systems*, *Purdue Growth Symposium*, pp. 93–106. New York: Basic Books Inc.

In the above case, 25–30% of the total protein of the 'super-constitutive' mutant appeared to be β-galactosidase. This mutant is, in fact, extremely vulnerable, competitively, under any other form of growth limitation since then the overproduction of the enzyme would be a waste of energy. For this reason, if this culture is transferred to glucose limitation, other mutants, which cannot be distinguished from the original (wild type) parent strain, will rapidly appear. In summary, the striking changes shown in the final mutant demonstrate the extreme selective forces that can be exploited in the chemostat to obtain mutants that have taken advantage of the chosen limitation. For a representative set of other examples the reader is referred to the reviews by Kuenen and Harder and Sikyta.

Egli's team has studied the selection of mutants of *E. coli* in glucose-limited chemostats. With a combination of highly sensitive analytical tools for residual sugar concentrations as well as the availability of tools for physiological screening, a thorough analysis has been made of the events taking place during the selection of *E. coli* mutants under glucose limitation in the chemostat. It appeared that the selection events were consistent with Monod kinetics. Consecutive mutants, with higher affinity (i.e., a mutation leading to either higher μ_{max} or lower K_S) for glucose, gradually took over in the chemostat, as evidenced by the stepwise drop in the residual glucose concentration in the culture. Initially $\mu_{\max}$ improved, but this stopped after the first 150 h and then further improvements were primarily due to the lowering of the K_S. The experiments were reproducible in cultures with high number of cells in the culture (10^{11}), that is, the stepwise improvement could be predicted. However at low numbers of total cells (10^7), the mutation frequency for advantageous mutations (estimated to be $1/10^7$ cell duplications) caused a stochastic behavior. Evidently, at 10^{11} cells per culture, in the order of 10^4 favorable mutants would have been present after the first generation and the system did not have to wait for favorable mutants to overgrow the existing population. Over the time span of 500 h in these experiments, no mutant types other than the high-affinity strains established themselves in the culture. They obtained no evidence for the establishment of secondary populations of mutants with a lower affinity for glucose, as was observed by Rosenzweig and colleagues (1994), after long-term selection at $D = 0.2\ \text{h}^{-1}$. In this experiment, a mixture of mutants had been selected with a distribution of tasks after 773 generations (2500 h). This mixed culture remained essentially the same for an additional 450 generations. One dominant mutant had the highest affinity for glucose but excreted glycerol and acetate, which were consumed by a second and third (satellite) mutant, respectively. Hence under the extreme and permanent selection conditions, the distribution of tasks in the breakdown of glucose ('resource partitioning') apparently was the most effective way to deal with the regulatory consequences of metabolic rearrangement in the respective mutants. Although the exact steady-state fluxes of glycerol and acetate in the chemostat were not known, it must be assumed that the second and third mutants, having the capacity for glucose metabolism, were both growing mixotrophically on glucose and glycerol or acetate, respectively.

Metabolic Engineering

The group of Pronk has recently constructed a xylose-fermenting *S. cerevisiae* strain for alcohol production from the pentose. They inserted a xylose isomerase from a fungus in this yeast, which was originally incapable of metabolizing xylose even under aerobic conditions. The xylose isomerase converts the sugar into xylulose, which can be metabolized by the yeast. Once this was accomplished, oxygen-limited and anaerobic batch enrichment in the presence of high-xylose concentrations led to the selection of mutants that grew faster on xylose and eventually they ended up with a mutant that grew anaerobically on xylose. Genetic engineering to add bottleneck enzymes of the pentose phosphate cycle yielded a mutant that could grow anaerobically on xylose at a rate of $0.09\ \text{h}^{-1}$. The organism showed, however, a strong diauxic behavior towards xylose in the presence of glucose, which was undesirable for application in the commonly available industrial sugar mixtures derived from processing of wood for paper production. Subsequent selection was then performed in an anaerobic chemostat culture under simultaneous limitation by a mixture of glucose and xylose. Progressive decrease of the residual xylose in the culture indicated the appearance of mutants. A strain was obtained with much improved xylose uptake kinetics and rate of metabolism of the pentose in the presence of glucose, even in batch culture. The authors concluded that the bottleneck in this case was primarily caused by uptake of xylose. This again shows how one can take advantage of the strong selective forces existing in nutrient-limited continuous cultures. Further improvement was accomplished by enrichment of mutants in anaerobic sequencing batch culture to select for faster growth. In the end, a mutant was selected with a $\mu_{\max} = 0.22\text{–}0.25\ \text{h}^{-1}$ in a mixture of (10%, w/v) glucose and (2%) xylose, in which sequential but effective-anaerobic consumption of xylose at a rate of 0.9 grams per gram biomass per hour occurred.

Given the demonstrated selective forces under nutrient limitation the maintenance of prolonged steady states is not advisable in laboratory studies aimed at studying the wild-type strain. It is advisable to switch dilution rates after each steady state and to start with a fresh culture (using an original new inoculum) after not more than 3–5 consecutive steady states have been investigated. Reproducibility of any particular steady state must be checked with independent cultures.

Other Interactions in Continuous Culture

When we consider competition for single or mixed substrates, we assume that there are no other interactions between the organisms other than competition for the growth-limiting substrate. Commensalism was briefly mentioned, and in its 'pure' form it is defined as an interaction involving the excretion of a substrate that is used by a second organism, without any further interaction. Examples are the use of fermentation products or products from an electron acceptor, such as nitrite from nitrate or sulfide from sulfate. Some of these may be toxic and in such cases the first organism may need the second to perform optimally. Secondary products such as vitamins or antibiotics may play a crucial role as well. This may lead to stable coexistence if an organism with the highest affinity for the limiting substrate requires the vitamin from an organism with lower affinity. In the case of an antibiotic, the cell density may determine whether or not coexistence is possible.

Mixed cultures of organisms may also be stable if one organism consumes a substrate toxic to the second. In this way, mixtures of aerobic and anaerobic organisms have been studied in oxygen-limited continuous culture. Oxygen is removed by the first organism, before the second can carry out its obligate anaerobic metabolism as has been demonstrated for a mixture of aerobic heterotrophs and sulfate reducers and even methanogens. More complex interactions include the degradation of xenobiotics by consortia of organisms, where one organism initiates the degradation of the compound, but cannot grow on that product. It needs a second organism to metabolize the first product and excrete a second product that can be used by the first. Examples are known in which four or five organisms maintain themselves in continuous culture in a tightly closed interactive network.

Essential Equipment in Continuous Culturing

From the preceding theoretical consideration, it is clear that the only fundamental design requirement of any continuous culture system is that the culture be kept growing by a continuous input of fresh medium that is balanced by the removal of culture fluid at the same rate. It is essential that the culture is ‘ideally’ mixed, because the theory assumes a totally homogeneous system. A great variety of continuous culture systems have been used over the years, with properly evaluated systems ranging in size from just a few milliliters from modified Hungate tubes that were used for radioactive tracer analysis of metabolic fluxes, up to a 1500 m^3 industrial system that is used for the nitrification step in wastewater treatment.

For all practical purposes, it is recommended to use a commercially available fermentor of 1–2 l working volume with a potentially high oxygen transfer capacity to ensure good aeration for aerobic cultivation. If the fermentor is built of glass and stainless steel, or from autoclavable oxygen-impermeable polymers, such a system can also be used for anaerobic continuous cultivation.

It is beyond the scope of this article to elaborate on the detailed design of these systems, but a summary of the important general considerations is included because they are important for the construction of most general-purpose research chemostats. Today many very well-designed chemostat systems are commercially available. In **Figure 12**, an example of a small bench scale ($\approx$0.5 l working volume) continuous culture system is shown. Some of its design characteristics are as follows:

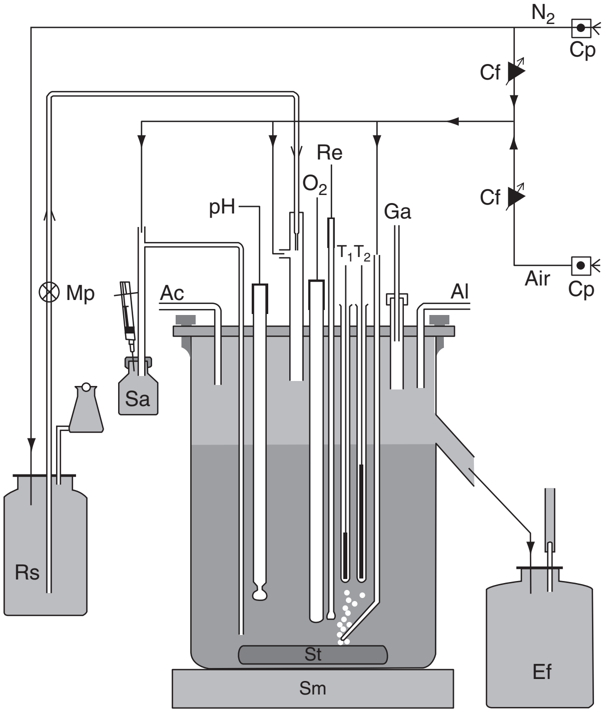

Figure 12 Schematic drawing of a small-scale (500-ml working volume), low-cost glass chemostat. All gases pass through cotton wool filters (not shown) before entering the fermentor. For anaerobic cultivation the N$_2$ is freed of traces of oxygen by passage over heated copper turnings. Rs, reservoir medium; Mp, medium supply pump; Sa, sampling bottle; Ac and Al, acid and alkaline titration inlets, respectively; pH, autoclavable pH electrode; O$_2$, autoclavable polarographic oxygen electrode; Re, redox electrode; T$_1$ and T$_2$, temperature sensor and heating element; Ga, sampling outlet for head-space gas analysis; Cf and Cp, constant flow and pressure regulators, respectively, for maintaining a stable (mixed) flow of N$_2$ and air over and through the culture; St and Sm, magnetic stirring bar and motor unit, respectively; Ef, effluent from the culture.

1. It can be used for both aerobic and anaerobic cultivation. For aerobic use, silicone seals and tubing are ideal. For anaerobic cultivation, seals and tubing are typically made from neoprene rubber or similarly impermeable materials, into which holes can easily be drilled to fit the desired assortment of tubing and probes.
2. Sterility is easily maintained because the entire unit can be autoclaved and mixing is done with a magnetic coupling between the motor and the stirring shaft, eliminating potential problems associated with the sealing of the stirring shaft through the lid of the chemostat. For simplicity **Figure 12** shows a stirring bar. However, in fermenters with larger working volumes (>>0.5 l) much more elaborate stirrers with shaft and impellers are required to ensure homogeneity.
3. The medium is supplied by means of a peristaltic pump using a durable rubber such as ismaprene or norprene for anaerobic cultivation. Silicone or any other compatible rubber can be used with aerobic cultures. The culture volume is maintained at a constant level by the removal of fluid at the same rate that it enters. In the example, an overflow is shown for simplicity. However, the drawback is that such devices may not remove representative materials from the culture. Therefore the best device is a second pump removing liquid from below the liquid surface and

which is activated by a sensor touching the surface of the culture.

4. In some cases, an appropriate low cost and/or simple continuous culture system may be used. It is essential, however, that such a system be designed appropriately for its intended purpose and thoroughly checked for adequate performance. For example, the use of potentially hazardous and/or expensive compounds, such as ^{14}C- or ^{13}C-labeled substrate, may require using very small working volumes. A simple test that is often overlooked in continuous culture studies and that can easily demonstrate whether the culture is actually limited by the expected substrate is to check the relation and the proportionality of $\bar{C}_X$ with C_{Si} at one chosen intermediate D, when it can be expected that $\bar{C}_S << C_{Si}$ (eqn [12]). A very good example is described by Sauer and colleagues, who used 17 ml Hungate tubes as chemostats to study the dependency of intracellular fluxes of metabolite as a function of specific growth rate. The validation concerned aeration sufficiency, and predicted linearity of the specific consumption rate with the dilution rate as well as biomass yield, residual glucose, and acetate as a function of D.
5. Other potential issues of chemostat design may arise from the use of highly volatile compounds in which an open gas phase may not be appropriate. The use of hydrophobic compounds can be problematic as they are often permeable in rubber tubing and rubber seals, and so proper attention toward chemical compatibility is essential. Most general-purpose chemostats are also insufficient for growth under pressure so the continuous cultivation of hyperthermophiles requiring elevated pressure typically must be done in chemostats whose exteriors are made exclusively from stainless steel. Insufficient stirring and aeration can easily become a problem in cultures grown at very high cell densities, especially in combination with high dilution rates. These can also become a problem at low cell densities if the volume of the culture is scaled up too high and these parameters should therefore be carefully considered when setting up any continuous culture system.
6. One detailed comment must be made on the design of the nutrient-medium inlet, which is of critical importance. The inlet must be able to deliver a regular flow of small droplets to the culture vessel (to minimize any effects of discontinuity in the supply of fresh medium) while simultaneously preventing contamination of the nutrient reservoir by the back growth of organisms in the culture vessel. This is typically accomplished by using a device through which medium droplets fall freely and directly into the culture fluid through a relatively wide glass tube that is kept dry on the inside by a continuous flow of sterile gas in the direction of the culture vessel. However if this device is used at low dilution rate and small culture volume, the drop-wise addition will cause dramatic fluctuations of the concentration of the limiting nutrient in the culture. As a result the coupling between catabolism and anabolism may no longer be optimal, which leads in turn to the low yield.

Important Aspects of Continuous Culture

1. Continuous culture in a chemostat enables the reproducible growth of bacteria and other microorganisms. Consequently, the chemostat is an appropriate tool for quantitative (eco)physiological research.
2. Microorganisms can be studied while growing at submaximal rates.
3. The effect of different growth limitations on the metabolism of the cells can be measured reproducibly.
4. The chemostat is also appropriate for studying the competition of microorganisms and mutants for growth-limiting substrates.
5. Continuous cultivation can also be performed under fluctuating environmental conditions.
6. Recent publications show the great value of chemostat cultivation for functional genomic research and for the selection of industrially relevant mutants.
7. The commercial availability of well-designed chemostat equipment greatly facilitates the introduction of continuous cultivation in the laboratory.

Acknowledgments

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Cyanobacteria

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Glossary

benthos Collection of organisms living on or within the sediments of water bodies.

monophyletic A taxon or group of organisms that contains all extant descendants of a common ancestor.

oxygenic photosynthesis Type of metabolism based on the coordinated action of two photosystems by means of which radiant energy is converted into chemical energy in the form of ATP, and reduction equivalents are obtained in the form of NADPH from the (photo)oxidation of water to free molecular oxygen.

phycobilisome Macromolecular aggregates serving as antenna systems for the capture of light in photosynthesis, typical of cyanobacteria. They are composed of multimers of different phycobiliproteins and of linker polypeptides. Phycobiliproteins are polypeptides containing covalently bound open tetrapyrroles chromophores: the phycobilins.

plankton Collection of organisms living suspended in open waters. According to their function, oxygenic phototrophic organisms in the plankton are termed phytoplankton. By an unorthodox convention, planktonic organisms smaller than 2 μm in size are called picoplankton.

sheath Structurally well defined, usually laminated, extracellular polysaccharide investment. The term is usually, but not exclusively, applied to those of filamentous cyanobacteria.

trichome Row of vegetative cells in filamentous cyanobacteria, excluding the extracellular polysaccharide structures (sheaths). The term is complemented by 'filament', which includes both the trichome and the sheaths.

Abbreviations

NADPH	nicotinamide adenine dinucleotide phosphate
PRK	phosphoribulosekinase
PSII	photosystem II
RubisCO	ribulose 1,5-bisphosphate carboxylase/ oxygenase
UV	ultraviolet

Defining Statement

Cyanobacteria are a monophyletic, ancient, morphologically diverse, and ecologically very important group of phototrophic bacteria that carry out water-oxidizing, oxygen-evolving, plant-like photosynthesis. With few exceptions, they synthesize chlorophyll *a* as major photosynthetic pigment and phycobiliproteins as light-harvesting pigments. They fix CO_2 as the sole source of carbon using primarily the reductive pentose phosphate pathway.

Introduction

Cyanobacteria constitute a phylogenetically coherent group of evolutionarily ancient, morphologically diverse, and ecologically important phototrophic bacteria. They are defined by their ability to carry out oxygenic photosynthesis (water-oxidizing, oxygen-evolving, plant-like photosynthesis). With few exceptions, they synthesize chlorophyll *a* as major photosynthetic pigment and phycobiliproteins as light-harvesting pigments. All are able to grow using CO_2 as the sole source of carbon, which they fix using primarily the reductive pentose phosphate pathway. Their chemoorganotrophic potential is restricted to the mobilization of reserve polymers (mainly glycogen) during dark periods, although some strains are known to grow chemoorganotrophically in the dark at the expense of external sugars. As a group, they display some of the most sophisticated morphological differentiation among the bacteria, and many species are truly multicellular organisms. Cyanobacteria have left fossil remains as old as 2000–3500 million years, and they are believed to be ultimately responsible for the oxygenation of Earth's atmosphere. During their evolution, through an early symbiotic partnership, they gave rise to the plastids of algae and higher plants. Today cyanobacteria make a significant contribution to the global primary production of the oceans and become locally dominant primary producers in many extreme environments, such as hot and cold deserts, hot springs, and hypersaline environments. Their global biomass has been estimated to exceed 10^{15} g of wet biomass, most of which is accounted for by the marine unicellular genera *Prochlorococcus* and *Synechococcus*, the filamentous genera *Trichodesmium* (a circumtropical marine form), as well as the terrestrial *Microcoleus vaginatus* and *Chroococcidiopsis* sp. of barren lands. Blooms of cyanobacteria are important features for the ecology and management of many eutrophic fresh and brackish water bodies. The aerobic nitrogen-fixing capacity of some cyanobacteria makes them important players in the biogeochemical nitrogen cycle of tropical oceans, terrestrial environments, and in some agricultural lands. Because of their sometimes large size, their metabolism, and their ecological role, the cyanobacteria were long considered algae; even today it is not uncommon to refer to them as blue-green algae, especially in ecological studies.

With the possible exception of their capacity for facultative anoxygenic photosynthesis, cyanobacteria in nature are all oxygenic photoautotrophs. It can be logically argued that after the evolutionary advent of oxygenic photosynthesis, the evolutionary history of cyanobacteria has been one geared toward optimizing and extending this metabolic capacity to an increasingly large number of habitats. This article provides an overview of the characteristics of their central metabolism and a necessarily limited impression of their diversity. Generalizations might, in the face of such diversity, easily become simplifications. Whenever they are made, the reader is reminded to bear this in mind.

Biodiversity: Taxonomy and Phylogeny

Taxonomy

For the newcomer, the taxonomy of cyanobacteria can easily become a nightmare; for the initiated, it is a persistent headache. Because in many cases cyanobacteria are indistinguishable ecologically from eukaryotic microalgae, they had been studied mostly by botanists. The epithets blue-green algae, Cyanophyceae, Cyanophyta, Myxophyceae, and Schizophyceae all apply to cyanobacteria. The botanical taxonomy was built using overwhelmingly morphological criteria based on observations from natural samples. Actually, two parallel botanical taxonomic treatments exist. The 'Geitlerian' system, recognizing approximately 1300 species grouped in 145 genera and three orders, has been the most widely accepted, and has been the subject of more modern updates and modifications in the 'Anagnostidis/Komarek' system. The Drouet system represented an attempt to arbitrarily simplify the previous one, recognizing only 62 species in 24 genera. It was never judged appropriate by most taxonomists, but it was welcome by many biochemists and physiologists because of its ease of use. Many names of laboratory strains stem from Drouet's system, such as *Anacystis nidulans*, the *Escherichia coli* of cyanobacteria. The prokaryotic nature of cyanobacteria began to be fully recognized in the 1970s, when a taxonomic system based on the International Code of Nomenclature of Bacteria was initiated. This system relies on the study of cultured axenic strains, and it draws heavily on morphological and cytological information, but integrates some genetic and physiological traits as well. The maximal taxonomic resolution so far achieved in this system is at the genus level. The current state of the bacteriological taxonomy of the cyanobacteria is described in the second edition of the *Bergey's Manual of Determinative Bacteriology*. Agreement exists that eventually both systems should converge, but to date they coexist with their own advantages and shortcomings. One is left with the choice of either using a system that allows identification of species but is largely unreliable or using a more reliable system in which species are yet to be defined. The diagnostic key to the cyanobacterial subsections (a taxon akin to Order), as given in the *Bergey's Manual*, is reproduced in **Table 1**. Most of the so-called form genera recognized within each subsection can be found in **Figure 1**, in which they have been gathered according to some simple, mostly visual keys. This unavoidably abridged overview is not intended as a substitute for more thorough generic descriptions. In this article, for consistency, taxa not recognized in the *Bergey's Manual* appear in quotation marks, without intended detriment to their validity.

Phylogeny

The most widely accepted reconstructions of cyanobacterial phylogeny are those based on comparisons of the 16S ribosomal RNA gene sequences. The salient traits of cyanobacterial ribosomal phylogeny have found support when other genes or multilocus analyses have been carried out, even with large sets of genes. Currently, several characteristics of cyanobacterial evolution can be

Table 1 Diagnostic key to the subsections of the cyanobacteria, according to the *Bergey's Manual of Determinative Bacteriology*

Subsection (traditional order)	*Definitory criteria*
Subsection I (Chroococcales)	Unicellular, nonfilamentous. Cells occurring singly or in aggregates. Cell division by binary fission in 1, 2, or 3 planes, symmetric or asymmetric, or by budding
Subsection II (Pleurocapsales)	Unicellular, nonfilamentous. Cells occurring singly or in aggregates. Reproduction by multiple fission without growth, yielding beaocytes (cells smaller than the parent cell), or by binary and multiple fission
Subsection III (Oscillatoriales)	Filamentous, binary fission in one plane, yielding uniseriate trichomes without true branching. No heterocysts or akinetes formed
Subsection IV (Nostocales)	Filamentous, division occurring only in one plane to yield uniseriate trichomes without true branching. Heterocysts formed when combined nitrogen is low
Subsection V (Stigonematales)	Filamentous, division occurring periodically or commonly in more than one plane, yielding multiseriate trichomes, truly branched trichomes, or both. Heterocysts formed when combined nitrogen is low

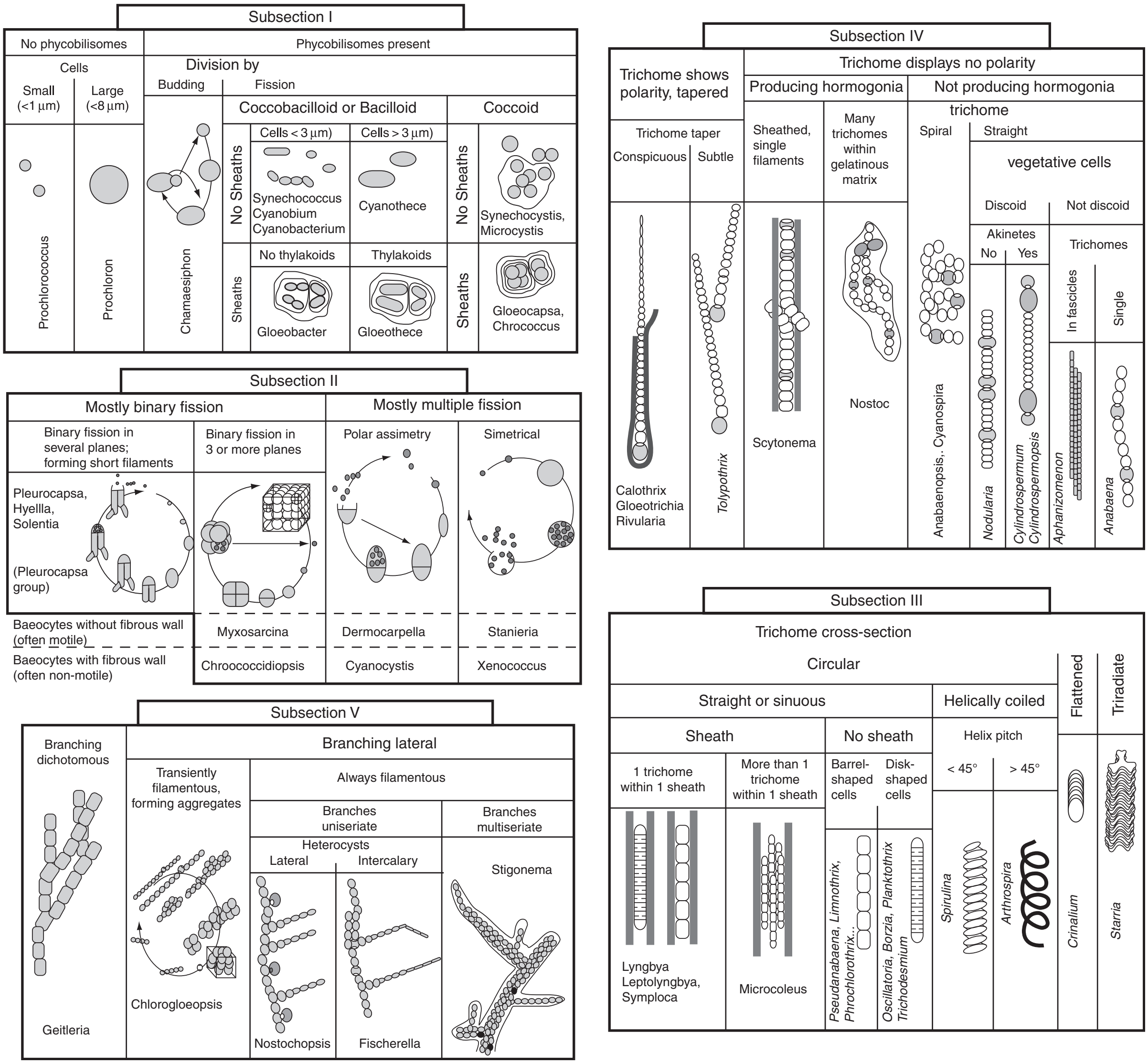
Subsection I
No phycobilisomes
Cells
Small (<1 μm)
Large (<8 μm)
Prochlorococcus
Prochloron
Phycobilisomes present
Division by
Budding
Fission
Chamaesiphon
Coccobacilloid or Bacilloid
Coccoid
No Sheaths
Cells < 3 μm)
Cells > 3 μm)
Synechococcus
Cyanobium
Cyanobacterium
Cyanothece
Synechocystis,
Microcystis
Sheaths
No thylakoids
Thylakoids
Gloeobacter
Gloeothece
Gloeocapsa,
Chrococcus
Subsection II
Mostly binary fission
Binary fission in several planes; forming short filaments
Binary fission in 3 or more planes
Pleurocapsa,
Hyellla,
Solentia
(Pleurocapsa group)
Mostly multiple fission
Polar assimetry
Simetrical
Baeocytes without fibrous wall (often motile)
Baeocytes with fibrous wall (often non-motile)
Myxosarcina
Dermocarpella
Stanieria
Chroococcidiopsis
Cyanocystis
Xenococcus
Subsection V
Branching dichotomous
Geitleria
Branching lateral
Transiently filamentous, forming aggregates
Chlorogloeopsis
Always filamentous
Branches uniseriate
Heterocysts
Lateral
Intercalary
Nostochopsis
Fischerella
Branches multiseriate
Stigonema
Subsection IV
Trichome shows polarity, tapered
Trichome taper
Conspicuous
Subtle
Calothrix
Gloeotrichia
Rivularia
Tolypothrix
Trichome displays no polarity
Producing hormogonia
Sheathed, single filaments
Scytonema
Many trichomes within gelatinous matrix
Nostoc
Not producing hormogonia
trichome
Spiral
Anabaenopsis,. Cyanospira
Straight
vegetative cells
Discoid
Akinetes
No
Yes
Nodularia
Cylindrospermum
Cylindrospermopsis
Not discoid
Trichomes
In fascicles
Single
Aphanizomenon
Anabaena
Subsection III
Trichome cross-section
Circular
Straight or sinuous
Sheath
1 trichome within 1 sheath
Lyngbya
Leptolyngbya,
Symploca
More than 1 trichome within 1 sheath
Microcoleus
No sheath
Barrel-shaped cells
Pseudanabaena, Limnothrix,
Phrochlorothrix..
Disk-shaped cells
Oscillatoria, Borzia, Planktothrix
Trichodesmium
Helically coiled
Helix pitch
< 45°
> 45°
Spirulina
Arthrospira
Flattened
Crinalium
Triradiate
Starria

Figure 1 Genera (form genera) recognized in the *Bergey's Manual of Determinative Bacteriology* within each of the five subsections of the cyanobacteria. Genera have been arranged according to the most important (abridged) definitory criteria, and drawings are provided depicting their simplified morphological appearance and/or their life cycles.

inferred, and some taxonomic controversies have been settled. However, several apparent paradoxes have also been unveiled. Extreme caution should be exercised in the interpretations of phylogenetic trees of cyanobacteria because the nomenclatural chaos has already pervaded the molecular databases. In **Figure 2**, a phylogenetic tree for the cyanobacterial radiation is presented. The cyanobacteria are a diverse phylum within the bacterial radiation, well separated from their closest relatives. The trees support clearly the endosymbiotic theory for the origin of plant chloroplasts because they place plastids (from all eukaryotic algae and higher plants investigated) in a very diverse but monophyletic deep-branching cluster. However, the gross structure in the evolutionary history of extant cyanobacteria cannot be easily resolved; most of the cyanobacterial diversity probed seems to be

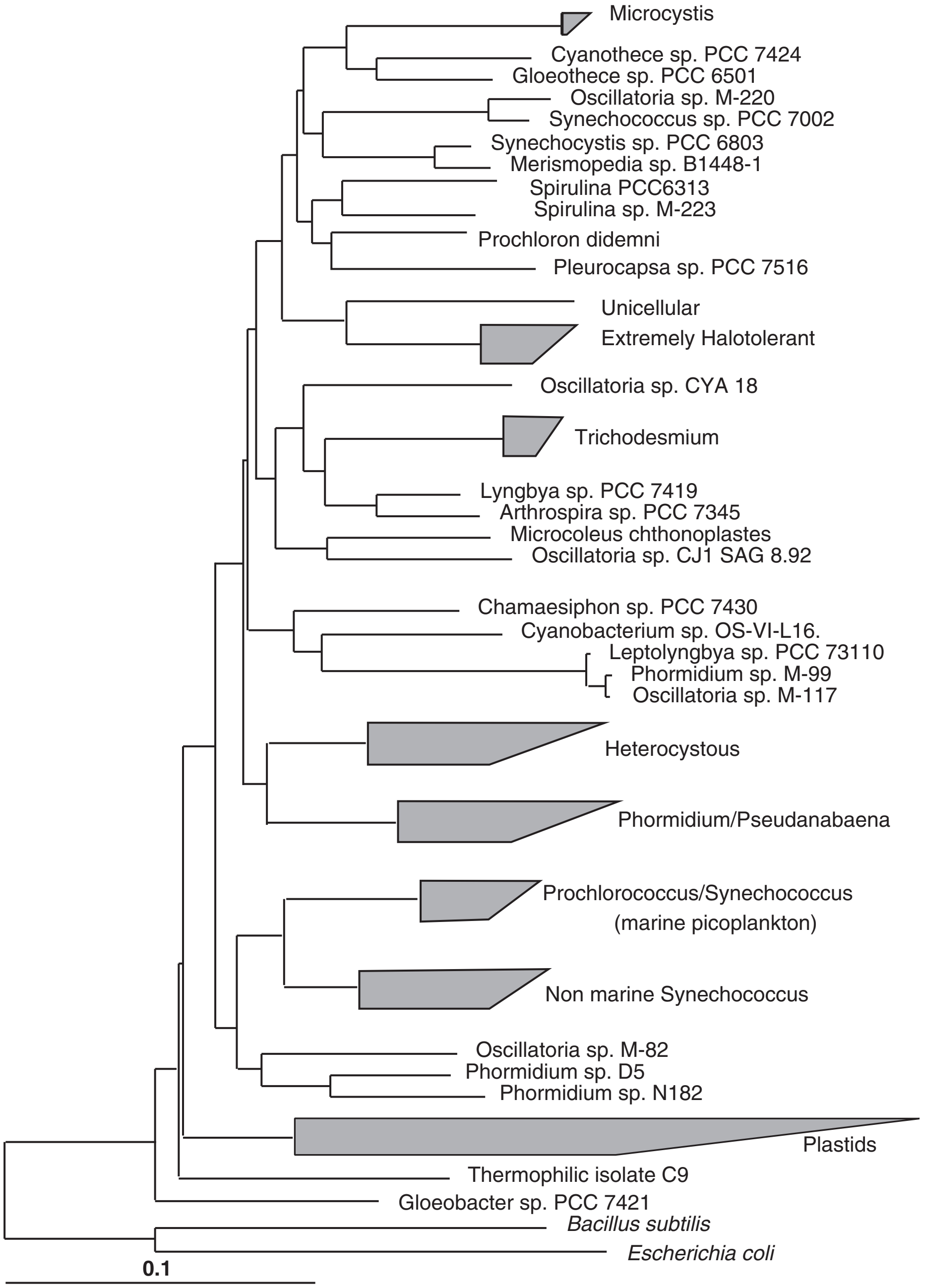
Microcystis
Cyanothece sp. PCC 7424
Gloeothece sp. PCC 6501
Oscillatoria sp. M-220
Synechococcus sp. PCC 7002
Synechocystis sp. PCC 6803
Merismopedia sp. B1448-1
Spirulina PCC6313
Spirulina sp. M-223
Prochloron didemni
Pleurocapsa sp. PCC 7516
Unicellular
Extremely Halotolerant
Oscillatoria sp. CYA 18
Trichodesmium
Lyngbya sp. PCC 7419
Arthrospira sp. PCC 7345
Microcoleus chthonoplastes
Oscillatoria sp. CJ1 SAG 8.92
Chamaesiphon sp. PCC 7430
Cyanobacterium sp. OS-VI-L16.
Leptolyngbya sp. PCC 73110
Phormidium sp. M-99
Oscillatoria sp. M-117
Heterocystous
Phormidium/Pseudanabaena
Prochlorococcus/Synechococcus
(marine picoplankton)
Non marine Synechococcus
Oscillatoria sp. M-82
Phormidium sp. D5
Phormidium sp. N182
Plastids
Thermophilic isolate C9
Gloeobacter sp. PCC 7421
Bacillus subtilis
Escherichia coli
0.1

Figure 2 Phylogenetic tree of the cyanobacteria based on (complete) 16S ribosomal RNA sequences. Scale at bottom represents genetic distance. Vertical distances bear no meaning. Clusters of (more than two) sequences that were well supported statistically have been collapsed into polygonal boxes of fixed width. The distance contained within each cluster is represented by the difference in horizontal length of opposing sides.

due to an explosive radiation that took place early during their evolution. It seems clear that the current taxonomic treatment of the cyanobacteria diverges considerably from a natural system that reflects their evolutionary relationships. For example, subsections I, III, and perhaps also II (orders Chroococcales, Oscillatoriales, and Pleurocapsales, respectively) do not find support in the trees as coherent units. The heterocystous cyanobacteria (subsections IV and V; Nostocales and Stigonematales, respectively) together form a monophyletic group with relatively low sequence divergence, but they are not monophyletic in their own right. Several other statistically well-supported groups of strains can be distinguished that may or may not correspond to currently defined taxa. The *Microcystis* (see 'Gene transfer') of unicellular colonial freshwater plankton species is very well supported by phylogenetic reconstruction, as is the genus *Trichodesmium* (see 'Genomes') of filamentous, non-heterocystous nitrogen-fixing species typical from oligotrophic tropic marine plankton. A grouping not corresponding to any official genus, the *Halothece* cluster, is composed of unicellular strains that are extremely tolerant to high salt and stem from hypersaline environments. A second grouping, bringing together very small unicellular open-ocean cyanobacteria (picoplankton: see 'Genomes'), includes members of the genera *Synechococcus* and all of *Prochlorococcus*. The picture that seems to emerge from these studies is that ecology and physiology are extremely important parameters to understanding the phylogenetic relationships of cyanobacteria and to achieving an evolutionarily coherent taxonomic system. Reaching this goal will necessitate continued efforts in the future.

Cytology and Morphogenesis

Cytology and Ultrastructure

Cyanobacterial cells range in width between 0.5 μm (e.g., *Prochlorococcus*) and 50–100 μm (e.g., some '*Chroococcus*' and some *Oscillatoria*); the modal size of the described species is significantly larger than that of most other bacteria and archaea (~4 μm). In unicellular and colonial forms, cells may be spherical, bacilloid, or fusiform, and some strains present considerable pleiomorphism. Cells of filamentous cyanobacteria may range from discoid to barrel-shaped, and the trichomes often attain lengths on the order of millimeters. The filamentous genus *Starria* has triradiate cells. Several types of cells may be present in morphologically complex cyanobacteria. The cells of most cyanobacteria are surrounded by a more or less-defined exopolysaccharide investment. In some species this may form a distinct, structured capsule or sheath, where the steric constrictions to cell growth imposed by the presence of mechanically strong capsules or sheaths may even dictate cellular shape (**Figure 3(p)**). Several ultrastructural features are typical for cyanobacteria. The cell envelope is of a Gram-negative type but may attain a considerable thickness in the peptidoglycan layer (from several to 200 nm). Pores of different sizes, whether or not orderly arranged, perforate the cyanobacterial cell wall. Pore pits may allow close contact of the cytoplasmatic membrane with the lipopolysaccharide outer membrane. The photosynthetic machinery of cyanobacteria resides on intracellular membranes (thylakoids). Each thylakoid consists of a double-unit membrane enclosing an intrathylakoidal space (**Figure 4(d)**). Thylakoids may be arranged parallel to the cell membrane, radially, or in small disorderly stacks, depending on species (**Figure 4**). They may be single or stacked, usually depending on illumination conditions. *Gloeobacter* has no thylakoids and the photosynthetic apparatus resides in the plasma membrane. The phycobilisomes, used by most cyanobacteria as light-harvesting structures, can be distinguished in electron microscopic preparations as rows of particles ~50 nm in diameter on the cytoplasmatic side of the thylakoids (**Figure 4(e)**). These are obviously absent from chlorophyll *b*-containing, phycobiliprotein-lacking species (*Prochlorococcus*, *Prochloron*, and *Prochlorothrix*). A central electron-clear region in the cell, the nucleoplasm, or the centroplasm, hosts the cellular DNA. Several intracellular nonmembrane-bound granules typically correspond to polymeric reserve materials, such as glycogen (polyglucose, usually present in the intrathylakoidal space), polyphosphate, poly-β-hydroxyalkanoates, and lipid droplets found in the cytoplasm proper, which are also common in other bacteria (**Figure 4**). Cyanophycin (multi-L-arginyl-poly(L-aspartic acid)) is a cytoplasmatic, exclusively cyanobacterial nitrogen reserve polymer. Carboxysomes (or polyhedral bodies) are commonly seen as membrane-bound intracellular inclusions, and they consist of accumulations of the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), responsible for the initial carboxylation step in the Calvin cycle (see 'Dark reactions of photosynthesis: carbon fixation and uptake'), bound by a proteinaceous, viral-capside-like case (**Figure 4(e)**). Gas vesicles are air-filled, cylindrical proteinaceous structures present in many planktonic species and in the dispersal stages of benthic forms; they provide buoyancy to the organisms. A large variety of ultrastructural inclusions of restricted occurrence are also known from particular species or strains.

Growth and Cell Division

Some strains cultured under optimal conditions display doubling times as short as several hours. In nature, one or more doublings per day is common in blooming planktonic populations, and cell divisions may be

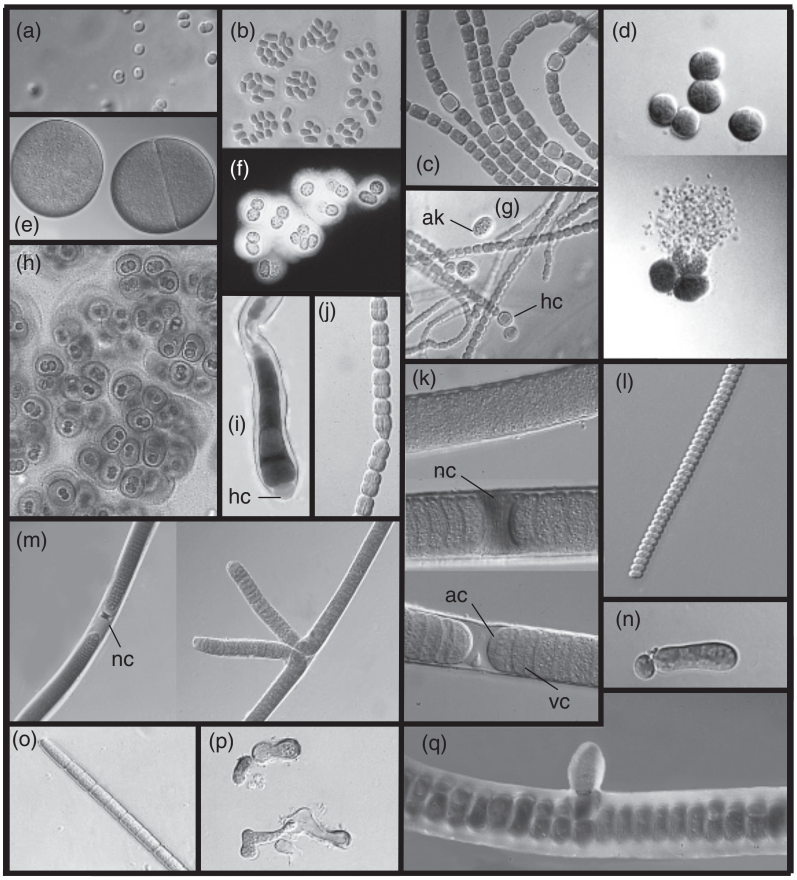

Figure 3 Aspects of morphological diversity and morphogenetic processes in cyanobacteria. (a) Coccoid cells of *Synechocystis* sp. strain PCC 6803, one of the most widely studied cyanobacteria. (b) Loosely aggregated colonies of a unicellular, extremely halotolerant cyanobacterium belonging to the Halothece cluster. (c) Filamentous *Anabaena* with intercalary heterocysts (hc). (d) Formation and extrusion of baeocytes in unicellular colonial cyanobacteria after repeated rounds of division without growth (sequence from top to bottom). (e) *Chroococcus turgidus*, the largest unicellular cyanobacterium known with right cell undergoing fission. (f) Negative stain (India ink) of a colony of *Gloeothece* sp., rendering the diffuse extracellular polysaccharide investments visible. (g) Cell differentiation in *Nostoc* showing vegetative barrel-shaped cells, hetrocysts (hc), and akinetes (ak). (h) Field sample of *Gloeocapsa sanguinea*, with red-stained, well-structured extracellular polysaccharide sheaths. (i) A young filament of *Calothrix*, displaying a terminal heterocyst (hc) and a tapering trichome. (j) Filament of a *Pseudanabaena*-like, *Konvophoron*, with deep constrictions at cross walls and displaying trichome division by direct differentiation of mamillated (nipple-bearing) apical cells without mediation of necridia. (k) Trichome division in a *Lyngbya*-like (*Porphyrosiphon*) filamentous strain. The top-to-bottom sequence displays the formation of necridial cells (nc), their degradation, and the formation of two rounded apical cells (ac), different from the discoid intercalary cells and a leftover vestigial cell. (l) Hellically coiled filament of *Spirulina*. (m) Heterocystous *Scytonema* showing the formation of false branches, as recently divided trichomes (see vestigial necridium, nc) break free from the sheath and continue to grow (not glide). (n) Asymmetric division (budding) in unicellular cyanobacteria of the genus *Chamaesiphon*. (o) Single trichome of *Microcoleus chthonoplastes*, with slight constrictions at the cross walls and bullet-shaped apical cell. (p) Pleiomorphy in a unicelular cyanobacterium, *Cyanophanon*, morphology being a result of steric constrictions to growth by their tough sheath. (q) Biseriate filament of *Stigonema*, with a true lateral branch.

strongly governed by daily periodicity. Under extreme conditions in the natural environment, net growth is sometimes best measured in units of percentage increase in biomass per year. Many cyanobacteria owe their ecological dominance and success not so much to the fast growth as to their ability to grow slowly and steadily in the face of environmental adversity, and slow growth rates may be an integral, genetically fixed part of the biology of many cyanobacteria. Cyanobacteria cells divide basically by fission, but the patterns of cell division, which vary, form the base of a morphological diversity unparalleled among prokaryotic organisms (**Figure 1**). It is surprising that, because morphological complexity is such a unique cyanobacterial trademark in the bacterial world, our knowledge of the regulation of cell division patterns and morphogenetic implications at the biochemical and molecular level is virtually nonexistent. The extracellular slime layers or sheaths may contribute considerably to morphogenesis in some cyanobacteria by simply holding cells together after division in disorderly colonies (as in *Microcystis*), by holding filaments together into

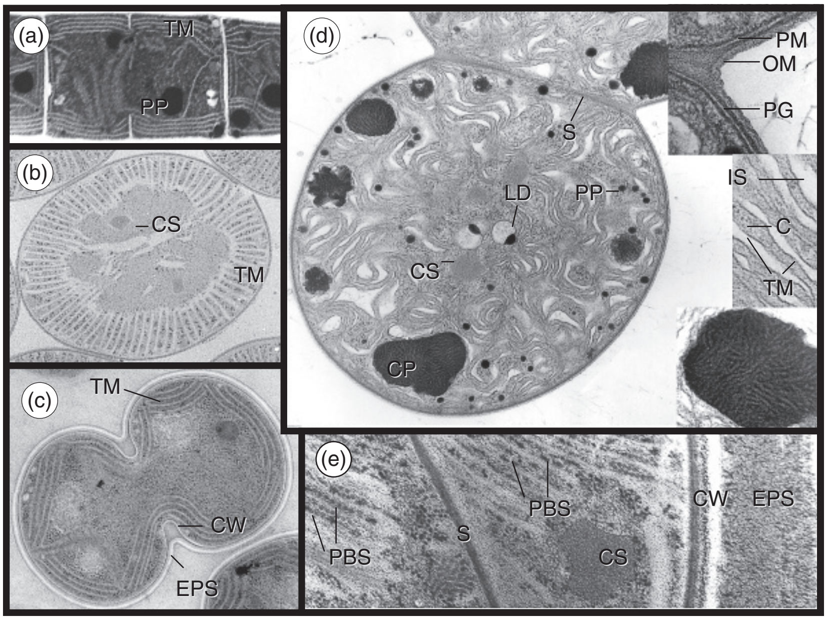

Figure 4 Cyanobacterial ultrastructure as seen by transmission electron microscopy. (a) Longitudinal section through a trichome of *Oscillatoria* '*lacus-solaris*', showing thylakoid membranes parallel to the cell wall (TM), and large polyphosphate (PP) granules, as well as incipient septa (cross-walls). (b) Trasversal section through a trichome of *Microcoleus chthonoplastes* showing radial, peripheral arrangement of thylakoids, a clear centrosome containing carboxysomes (polyhedral bodies, CS). (c) Dividing *Synechocystis* strain PCC6803, showing clearly equatorial invagination of all tegumentary layers including exopolysaccharide (EPS) and cell wall (CW), and parallel sets of thylakoids membranes. (d) End cell in a *Nostoc pruniforme* trichome, with characteristic cyanophycin granules (CP), carboxysomes (CS), polyphosphate granules (PP), unordered arrangement of thylakoids, lipidic droplets (LD), and septum or cross wall (S). The top insets show a detail of the tegumentary layers around the cross wall constrictions, with the plasma membrane (PM), and the peptidoglycan wall (PG) entering to form the septum, to the exclusion of the outer membrane (OM). The middle inset shows a thylakoid-rich region with thylakoid membranes (TM), intrathylakoidal space (IS), and the cytoplasm (C). The bottom inset shows a cyanophycin granule. (b) Outer regions in a *Oscillatoria* filament with, from right to left, exopolysacharide (EPS) layer, cell wall (CW), a carboxysome (or polyhedral body, CS), rows of phycobilisomes (PBS) sitting on the cytoplasmatic side of the thylakoid membranes, and a septum (S). After unpublished photomicrographs from Mariona Hernández-Mariné (University of Barcelona, all but (c)) and Robert Roberson (Arizona State University, (c)), with permission.

bundles (as in *Microcoleus*), or by allowing a localized extrusion of trichomes that result in 'false branching' (as in *Scytonema*; **Figure 3(m)**).

Unicellular and colonial types

Cell division occurs here by inward growth of all tegumentary structures (cytoplasmatic membrane, cell wall, outer membrane, and slime sheath), usually at an equatorial position (**Figure 4(c)**). Asymmetrical division in one pole of unicells may result in small-sized daughter cells (budding; **Figures 3(n)** and **3(p)**), as in *Chamaesiphon*. A genetic capacity to alternate between two or three orthogonal division planes yields spatially ordered colonial forms, as in *Merismopedia* (planar colonies – two alternating division planes) or in *Myxosarcina* (cubical colonies – three alternating division planes). Multiple divisions occurring in the absence of cell growth result in the formation of a multiplicity of minute daughter cells (baeocytes) that eventually break free from the cell wall remains of the parental cell, as in all 'pleurocapsalean' (subsection III) cyanobacteria (**Figure 3(d)**).

Filamentous types

Repetitive division in a single plane without complete cell separation yields simple filamentous forms. The filamentous nature of some cyanobacteria may simply be due to this fact and may not necessarily imply a functional integration of the cells into a truly multicellular organism. In morphologically complex filamentous forms, however, the outer tegumentary layers may be continuous along the trichome, and the formation of cross walls or septa may involve the invagination of the plasma membrane and the cell wall only, as has been shown in *Nostoc* (**Figure 4(d)**). In filamentous oscillatorian cyanobacteria, one or more tegumentary invaginations (future cross walls) may be initiated before cell division is completed. A widespread change of division plane in the cells along the trichome results in biseriate or multiseriate trichomes (two or more rows of cells), and a change in the plane of division occurring in a single cell and maintained for several rounds of fission results in true branching, as in *Stigonema* (**Figure 3(q)**). In morphologically complex filamentous forms, cell division may be meristematic, occurring only in certain portions of the trichome (e.g., *Calothrix* and allied forms).

Multicellularity and Cell Differentiation

The magnitude of the morphological complexity evolved within the cyanobacterial radiation is exemplified by the achievement of multicellularity. The heterocystous group (subsections IV and V) and some oscillatorians are clearly not mere linear arrays of cells but truly multicellular organisms, possessing all the attributes required for such a distinction: supracellular structural elements, integrated behavioral responses to environmental stimuli, and distribution of labor through cell differentiation into distinct cellular types. The most important examples of cellular differentiation in cyanobacteria are reviewed here. Additionally, in many cases cyanobacteria form colonies or structurally organized macroscopic bodies (thalli in botanical terminology) that are quite large in size. Spherical colonies of *Nostoc* '*pruniforme*' exceeding 15 cm in diameter are known from the benthos of oligotrophic cold springs, but somewhat smaller, ordered arrangements of cells and filaments are common in other *Nostoc* and '*Aphanothece*' species as well as in *Calothrix* and allied genera (e.g., '*Rivularia*'). This metadifferentiation is seldom achieved in culture.

Hormogonia

Hormogonia (s. hormogonium) are short (~5–25 cells) chains of cells formed and released from the parental, larger trichome. They serve a function in the dispersal of the organism. Hormogonial cells may or may not be different in size and shape from vegetative cells. Detachment may involve the differentiation of a necridic cell separating the vegetative trichome from the hormogonium. Dispersal is aided by the expression of phenotypic traits, which may vary according to strains, such as gliding motility, development of gas vesicles, or change in surface hydrophobicity. Hormogonia eventually settle and dedifferentiate into a typical vegetative organism.

Heterocysts

Heterocysts (**Figures 3(c)**, **3(g)**, and **3(i)**) are morphologically distinct cells that develop in response to a lack of combined nitrogen sources in the environment. The ability to develop heterocysts occurs without exception within a monophyletic group of filamentous cyanobacteria (heterocystous; subsections IV and V). They are usually larger than vegetative cells, develop thick tegumentary layers and intracellular hyaline buttons at the points of attachment to the vegetative cells, displaying a pale coloration and reduced autofluorescence. As such, heterocysts are easy to recognize under the microscope. They may differentiate from end cells (terminal heterocysts, as in *Calothrix*) or from cells within the trichome (regularly spaced intercalary heterocysts, as in *Anabaena*, or lateral as in '*Mastigocoleus*'). Heterocysts are highly specialized in the fixation of dinitrogen under aerobic conditions. They represent a successful solution to the nontrivial problem of avoiding nitrogenase inactivation by free oxygen in oxygen-evolving organisms. Heterocyst biology has been relatively well studied at the biochemical and molecular levels. Heterocysts are the only cells that express *nif* (nitrogen fixation) genes and synthesize nitrogenase in heterocyst-forming cyanobacteria. Heterocysts do not evolve oxygen themselves (photosystem II (PSII) activity is absent or restricted) but a functional photosystem provides ATP. The source of reductant for nitrogen fixation is provided (as organic carbon) by the adjacent vegetative cells, which in turn obtain fixed nitrogen from the heterocyst in the form of amino acids (mostly glutamine). The heterocysts protect their nitrogenase from oxygen inactivation by maintaining reduced internal partial pressures of oxygen, a situation that is attained by means of increased rates of cellular respiration and, apparently, by restricting diffusive entry of oxygen from the environment as a result of their thick envelope. The developmental regulation of heterocysts is beginning to be understood at the genetic level. The autoregulated gene *hetR*, which is activated by the deficiency in combined nitrogen, seems to play a crucial role in the initiation of heterocyst development.

Akinetes

These nonmotile cells (**Figure 3(g)**) are characterized by their enlarged size with respect to vegetative cells, their thick cell wall and additional tegumentary layers, and their high content of nitrogen reserves in the form of cyanophycin granules. They are formed exclusively by heterocystous filamentous cyanobacteria (but not by all) and they may differentiate *en masse* or at special locations within the filaments (usually close to or next to a heterocyst). Because akinetes are resistant to desiccation, low temperatures (including freeze–thaw cycling), and digestion in animal guts, they are considered resting stages in the life cycle, but they fundamentally different from typical bacterial spores in that they are not heat-resistant. In natural planktonic populations, massive akinete formation occurs at the end of the growth season. Germination of akinetes occurs when the environmental conditions become favorable for the growth of a vegetative filament. Genetic evidence suggest that the early regulatory process of akinete development and that of heterocysts has a common basis.

Terminal hairs

These are multicellular differentiations occurring at the tips of trichomes in some members of the genus *Calothrix* and allied cyanobacteria (botanical family Rivularaceae). In response to nutrient limitation (e.g., phosphorous), the terminal parts of the trichome differentiate irreversibly into thin and long rows of narrow, almost colorless, vacuolated cells (hence the term hair). The hair is a site of preferential expression of cell surface-bound phosphatase activity.

Necridic cells (necridia)

Necridic cells occur in truly multicellular cyanobacteria (**Figures 3(n)** and **3(k)**). Necridic cells undergo a suicidal process (apoptosis), which begins with the loss of turgor and leakage of some cellular contents and continues with shrinkage and the separation of the cross walls (septa) from the adjoining cells. Eventually, the necridic cells will either rupture and disintegrate or remain as small, isolated vestigial cells. Cells adjacent to the necridium will usually develop morphologies typical for terminal (apical) cells. The formation of necridia may lead to the separation of one trichome into two (proliferation) or in the detachment of hormogonia from the vegetative filament. Most of the information about necridia is observational, and no studies have been performed to investigate the regulation of this morphogenetic mechanism.

Physiology and Metabolism

Cyanobacteria are photoautotrophic organisms *par excellence* and their metabolism is typically geared toward anabolic reactions. The basis of their metabolism is the conversion of radiant energy into chemically usable energy and the reduction of CO_2 into organic matter. The electron donor for the reduction of CO_2 is water, which is oxidized to molecular oxygen. The name of this type of metabolism is derived by the release of oxygen: oxygenic photosynthesis. It is exclusively carried out by organisms in the cyanobacterial radiation (cyanobacteria proper and the plastids of algae and plants).

Photosynthesis

In the light reactions of photosynthesis, radiant energy is captured and used to generate energy in the form of ATP and reduction equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH). The light reactions of oxygenic photosynthesis are based on the coordinated action of (1) light-harvesting systems; (2) two chlorophyll *a*-containing, membrane-bound, multisubunit enzymes known as photosystems; and (3) a series of soluble and membrane-bound electron-carrier proteins linking both photosystems (**Figure 5**).

Light harvesting

Light harvesting in most cyanobacteria (and in red algal plastids) is accomplished by highly ordered and structurally versatile supramolecular complexes known as phycobilisomes, which are primarily composed of

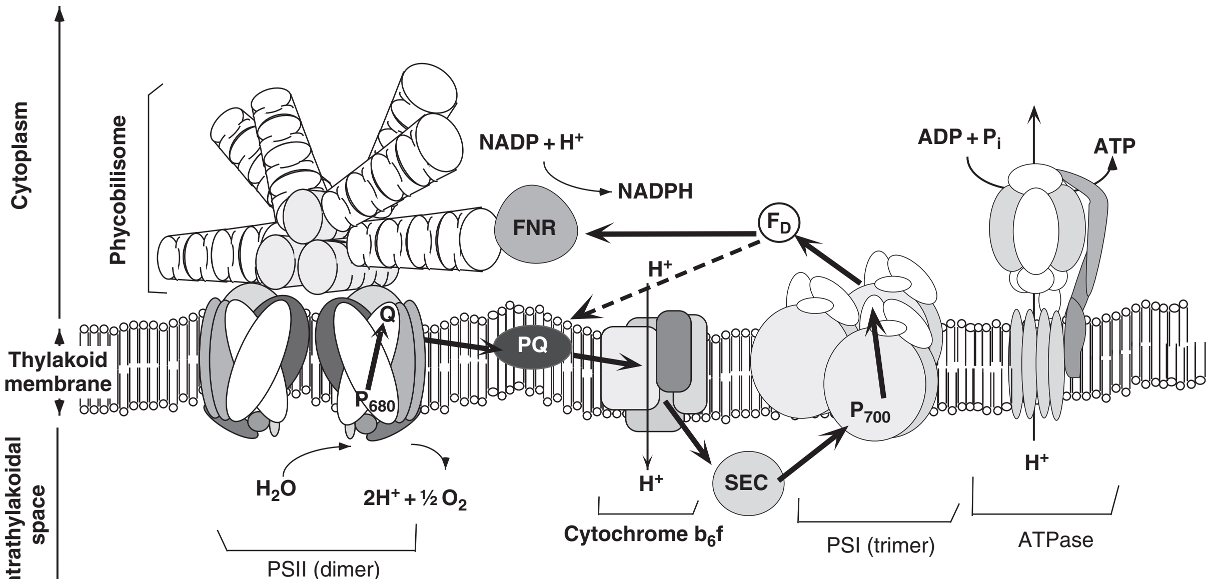

Figure 5 Idealized organization of the photosynthetic components in and around cyanobacterial thylakoids and their associated activities. Multimeric complexes are indicated by labeled brackets. Abbreviations not explained in text are bound quinone (Q), plastoquinone (PQ), soluble electron carrier (SEC), ferredoxin (FD), ferredoxin/NADP oxidoreductase (FNR). Thick black arrows indicate the direction of electron flow from water to NADPH; dashed arrow depicts the shortcut under cyclic phosphorylation conditions. Thin arrows depict either transformation of chemical reactants or the traffic of protons across the membrane. Modified from Bryant (1996), as cited, with permission.

phycobiliproteins. Phycobiliproteins are water-soluble proteinaceous pigments containing covalently bound, open-chain tetrapyrroles (phycobilines) as chromophores. Universal cyanobacterial phycobilines are the blue-colored allophycocyanin (absorbing maximally at a wavelength of 650 nm) and phycocyanin (maximum at 620 nm). Phycoerythrocyanin (maximum at 575 nm) and the red-colored phycoerythrin(s) (maxima vary from 495 to 560 nm) are also common. Multimeric disc-shaped phycobiliprotein complexes are stacked into either central cores or radially protruding rods (**Figure 5**) to form a functional phycobilisome. The radiant energy absorbed by the phycobiliproteins along the rods is channeled vectorially (as excitation energy) toward the core region and from the core onto the reaction center of PSII or (partially) onto that of photosystem I. Phycobiliproteins are arranged orderly within the phycobilisome: the shorter the wavelength of maximum absorbance, the more peripheral their location. This allows for the centripetal channeling of excitation energy down a thermodynamically allowed sequence. In such a light-harvesting system, 300–800 phycobilin chromophores capture additional energy for ~50 Chl *a* molecules associated with PSII. In some strains, the phycobilin composition of phycobilisomes can be regulated to optimize the capture of photons according to the color of the available light, a phenomenon known as complementary chromatic adaptation.

Light reactions of photosynthesis

PSII, which contains a reaction center, known as P680, of very high basal reduction potential (+1 V), catalyzes the transfer of electrons from water to a bound quinone, with the production of O_2. The electrons then enter an electron transport chain involving successive redox reactions of a membrane-bound protein (plastoquinone), a membrane-bound protein complex (cytochrome b_6f), and one of the two intrathylakoidal soluble electron carrier proteins (cytochrome c_{533} or plastocyanin). An electrochemical gradient of protons is created across the thylakoid membrane in the process of electron transport. This is used by the thylakoidal f-type ATPase complex to generate ATP, the cell's energy currency. When excited, PSI, with a reaction center (known as P700) of intermediate reduction potential, catalyzes the reoxidation of reduced plastocyanin (or cytochrome c_{533}) with the concomitant reduction of ferredoxin (a soluble iron–sulfur protein) against a steep thermodynamic gradient. Reduced ferredoxin is used by ferredoxin: NADP+ oxidoreductase (an enzyme physically tethered to phycobilisomes, if present) to generate the NADPH necessary for the dark reactions. In short, the light-driven formation of ATP and NADPH has been achieved. Additionally, electron flow around PSI alone may also occur (cyclic electron transport). In this case, electrons flow from reduced ferredoxin directly to plastoquinone, through the cytochrome b_6f complex and plastocyanin, back to PSI and, with light, to oxidized ferredoxin, closing the cycle. The net effect of the cycle is the generation of energy but no reductant.

Dark reactions of photosynthesis: Carbon fixation and uptake

The reduction of CO_2 to organic matter (carbon fixation) occurs in all cyanobacteria mainly through the reductive pentose phosphate (Calvin) cycle, in which the net formation of a triose from $3CO_2$ is powered by ATP and NADPH formed in the light reactions. This cycle supplies important intermediates for anabolic reactions (triose, pentose, and hexose phosphate). Additional CO_2 may be fixed by phosphoenolpyruvate carboxylase, yielding C_4 acids, and by carbamyl phosphate synthetase/carbamylphosphateornithine carbamyl transferase, yielding citrulline and glutamate from glutamine, ornithine, and CO_2. The Calvin cycle is related to the catabolic (oxidative) pentose phosphate pathway, differing in two key enzymes that allow it to function anabolically. These are PRK (phosphoribulosekinase) and RubisCO, a very interesting enzyme and the most abundant protein on Earth. RubisCO is characterized by a low affinity for CO_2 and by possessing internal monooxygenase activity. This results in a competitive inhibition of carboxylation by free oxygen, a fact of obvious importance for oxygen-producing phototrophs. Under conditions of low CO_2 and high O_2 partial pressure, RubisCO catalyzes the oxidation of ribulose bisphosphate to phosphoglycerate and phosphoglycolate. After dephosphorylation, glycollate is excreted by the cells in what seems to be a wasteful loss of carbon. Probably, to prevent conditions leading to such losses, cyanobacteria possess a carbon concentrating mechanism by which inorganic carbon, either as bicarbonate or as CO_2, is active and at the expense of energy transported into the cell so that the intracellular concentrations can be 1000-fold higher than those outside the cells. A carbonic anhydrase-like enzyme keeps intracellular carbon in the form of bicarbonate to prevent leakage of CO_2. A carbonic anhydrase located within the carboxysomes (or polyhedral bodies, the site of RubisCO accumulation; **Figure 4**) generates CO_2. With this system, a high CO_2 partial pressure is maintained locally in close proximity to the carboxylation sites of RubisCO, and the carboxylating activity of the enzyme is promoted.

Dark Metabolism

Energy generation in the dark occurs through aerobic respiration at the expense of glycogen accumulated during the light phase. Monomeric sugars are degraded using the oxidative pentose phosphate cycle. A complete tricarboxylic acid cycle has never been shown for – and α-ketoglutarate dehydrogenase has never been detected in – any cyanobacterium. NADPH formed in sugar catabolism is fed to the membrane-bound electron transport chain at the level of plastoquinone. Terminal oxidases are cytochrome oxidases of the aa_3 type. The respiratory electron transport chain of cyanobacteria is housed in both the plasma and the thylakoidal membrane and it shares many functional components with photosynthetic electron transport. Approximately half of all cyanobacterial strains tested are obligate phototrophs, unable to use exogenous carbon sources aerobically. Some are photoheterotrophs, able to use some sugars as carbon source, and some are facultative heterotrophs, able to grow, albeit slowly, at the expense of externally supplied sugars (usually only one) in the dark. All strains retain pigmentation and all components are necessary for photosynthesis under dark growth conditions. The lack of sugar transport systems has been heralded as one of the main reasons for the inability of many strains to use exogenous sugars while being able to respire endogenous glucose.

Cyanobacteria may also be subject to periods of anoxia, particularly in the dark (e.g., benthic forms thriving in sulfidogenic environments and biofilm or colony formers under diffusion limitations of O_2 supply). The only known electron acceptors alternative to oxygen for cyanobacterial chemoorganotrophy are internal organic compounds and elemental sulfur. Fermentation is not universal but is a relatively widespread ability in benthic and bloom-forming cyanobacteria. As in aerobic heterotrophy, fermentation occurs at the expense of endogenous sugars (usually glycogen but also sugar osmolytes such as trehalose or glucosylglycerol) accumulated in the light period. Some strains ferment, or even grow on, exogenous substrates anaerobically. Homolactic, heterolactic, homoacetate, and mixed-acid fermentation have all been described. There is evidence that the Embden–Meyerhof–Parnas glycolytic pathway, unoperative for aerobic respiration, is used in the fermentative degradation of sugars by cyanobacteria. An *Oscillatoria* strain oxidizes endogenous carbohydrates largely to CO_2 in the presence of elemental sulfur with the concomitant production of sulfide. In other cyanobacteria, sulfur may be used as a sink for electrons, otherwise released as H_2, with or without concomitant modification of the fermentative products. A thermophilic *Synechococcus* reduces sulfate and thiosulfate to sulfide anaerobically in the dark. It is yet to be demonstrated that the reduction of sulfur is coupled to electron transport or energy generation.

Secondary Metabolism

Cyanobacteria synthesize a variety of compounds that are not components of universal biochemical pathways, but have restricted distribution among taxa (secondary metabolites). These are thought to serve particular functions relevant to the survival of the strains in question, but their specific role has been deduced only in a few cases. Several important cyanobacterial metabolites are peptides synthesized in a nonribosomal setting by specific peptide synthetases. Compounds such as cyanobacterin (**Figure 6**(**c**)), a herbicide produced by some *Scytonema*, potently inhibit PSII of algae and cyanobacteria other than the producing strain, thus wiping out the competition. Scytonemin, a widespread indole alkaloid, is synthesized, excreted, and accumulated in large quantities in extracellular sheaths in response to ultraviolet (UV) radiation exposure, serving a sunscreen role (**Figure 6**(**h**)). A similar sunscreen role has been proposed for a large variety of colorless mycosporine-like compounds (**Figures 6**(**f**) and **6**(**g**)). Triterpenoids of the hopane series found in thermophilic strains may stabilize the cell membranes under high temperatures. Other compounds display antibiotic activity, such as the antibacterial brominated biphenyls from *Oscillatoria* '*chalybea*' (**Figure 6**(**f**)) or the methoxytetradecenoic acid of *Lyngbya* '*majuscula*' (**Figure 6**(**d**)). However, for most cyanobacterial secondary metabolites identified, their biological function remains elusive. Such is the case for the volatile compounds 2-methylisoborneol and geosmin (**Figures 6**(**a**) and **6**(**b**), respectively), which are of common occurrence and responsible for the earthy smell and off-flavors in lakes harboring cyanobacterial blooms. A defined biological role for the notoriously famous cyanotoxins (**Figures 6**(**i**) and **6**(**j**); see 'Gene transfer') is lacking. Among the bioactive compounds of unknown natural function, some have antineoplastic, antiviral, anti-inflammatory, antimitotic, ichtiotoxic, and dermatitic activities. Efforts to study the largely untapped cyanobacterial inventory of secondary metabolites and their biology are likely to increase substantially in the near future due to their relevance to pharmaceutical research and public health.

Nutrition

Apart from liquid water, light, and inorganic nutrients, few additional requirements for growth are known in most cultivated strains. A requirement for vitamin B_{12} has been demonstrated in some strains. Metabolic processes devoted to the provision of nutrients may account for a significant part of the energy and reduction equivalents obtained in the light reactions of photosynthesis. Cyanobacteria possess specific uptake systems for nutrient assimilation. Orthophosphate can be taken up and stored intracellularly as polyphosphate (**Figures 4**(**a**) and **4**(**d**)), and the uptake may be aided by the action of surface-bound phosphatases, which release phosphate bound to organic molecules. The availability of phosphorous may often be the

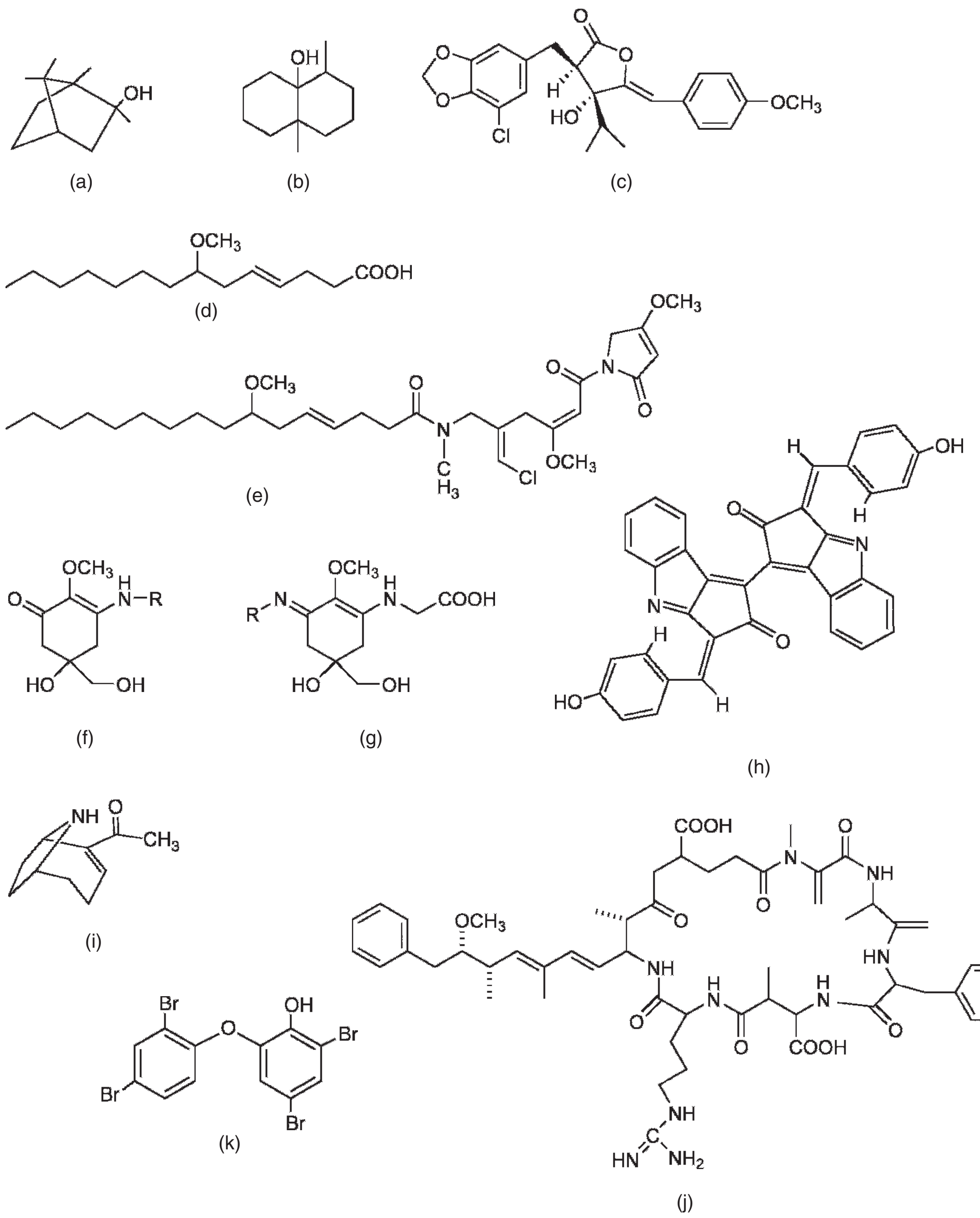

Figure 6 Diversity of cyanobacterial secondary metabolites. (a) 2-methylisoborneol; (b) geosmin; (c) cyanobacterin; (d) 7-methoxy-4-tetradecanoic acid; (e) malyngamide A; (f) and (g) mono- and bisubstituted mycosporines, respectively, where R stands for (amino) acidic moiety; (h) scytonemin; (i) anatoxin-a; (j) microcystin-YR; (k) a brominated phenyldiphenol.

growth-limiting factor in natural freshwater populations. The production of siderophores (iron-chelating organic compounds) seems to be important in the assimilation of iron because Fe^{3+} ions, required for many of the enzymes involved in redox reactions, are very insoluble in water. The availability of iron may be growth limiting in oceanic planktonic species. Nitrogen-fixing cyanobacteria have complex sets of amino acid uptake systems, probably geared toward the recovery of leaked fixed nitrogen. In addition to uptake mechanisms, sulfur, and nitrogen assimilation require additional reduction steps and are discussed separately in the following sections.

Nitrogen assimilation

Among inorganic nutrients, nitrogen is of paramount importance as it accounts for ~10% of the dry weight of cyanobacterial cells. Nitrate (NO_3^-) and ammonium (NH_4^+) are virtually universal sources of nitrogen for cyanobacteria, but urea or other organic nitrogenous compounds can be used by some strains. In addition, many strains can fix gaseous dinitrogen (N_2). Plasma membrane-bound transport systems exist for both NO_3^- and NH_4^+, whereas N_2 enters the cells by diffusion. Intracellular NO_3^- must be reduced to NH_4^+. This is accomplished by the stepwise reduction to nitrite (catalyzed by nitrate reductase) and NH_4^+ (catalyzed by nitrite reductase), the reduction equivalents for both processes stemming from reduced ferredoxin. Ammonium (either taken up or endogenously generated) is assimilated by the glutamine synthetase/glutamate synthase enzyme system. The net action of this system is the formation of glutamate from α-ketoglutarate and NH_4^+, with the expenditure of ATP and the oxidation of ferredoxin. Glutamate can donate its amino moiety to various precursors of central metabolism by the action of specific transaminases. Many, but not all, cyanobacteria are able to fix N_2; this is of great ecological significance because N_2 is ubiquitous in the environment. The process is carried out by the enzyme nitrogenase and is a costly one, involving the consumption of both ATP and reduction equivalents (supplied by ferredoxin). In addition, nitrogenase will also inevitably reduce protons to H_2 in what represents a wasteful decrease in efficiency. Nitrogenase is inherently and irreversibly inactivated by O_2. Several strategies have evolved in cyanobacteria to circumvent this problem. Some strains will only carry out N_2 fixation under anoxic conditions, but some will also do it in the presence of oxygen. Several strains have been shown to restrict temporally N_2 fixation to the dark period, thus decreasing the exposure of nitrogenase to photosynthetic oxygen. Strains belonging to the Nostocales and Stigonematales have evolved a specialized cell type (the heterocysts; see 'Heterocysts') in which nitrogen fixation is spatially separated from photosynthesis and protected from O_2 inactivation. Heterocystous strains display the highest specific rates of N_2 fixation among all cyanobacteria. However, some nonheterocystous cyanobacteria, such as *Trichodesmium*, are able to fix substantial, biogeochemically significant amounts of N_2 in the light; their mode of adaptation is unknown. The various mechanisms for nitrogen assimilation are tightly regulated so that the presence of less costly sources (NH_4^+) immediately inhibits NO_3^- (and NO_2^-) uptake, or N_2 fixation activity, and represses the expression of the enzymes involved in the reduction of alternative N_2 sources. In the same way, the presence of abundant NO_3^- represses the expression of nitrogenase genes and results in the halting of new heterocyst differentiation.

Sulfur assimilation

Sulfate (SO_4^{2-}) is seemingly the universal source of sulfur for cyanobacterial cells, and it is only rarely growth limiting in the environment. Other sources of sulfur may be taken up alternatively, such as sulfate esters, sulfonate, hydrogen sulfide, and organic thiols. Sulfate is taken up by a SO_4^{2-} permease in an energy-dependent process, reduced to sulfide, and incorporated into cysteine. The cyanobacterial assimilatory sulfate reduction pathway is similar to that of other bacteria, involving the activation of sulfate by binding to ADP and the reduction of the sulfonucleotide to free sulfite using thioredoxin as a reducing agent. Sulfite is further reduced to sulfide by sulfite reductase using NADPH as an electron donor, and free sulfide is incorporated into cysteine by specific synthases. An oxidized sulfur source may also be a requirement for growth because, unlike other bacteria, cyanobacteria possess important structural components containing oxidized sulfur moieties: the sulfolipids of the photosynthetic membranes and, in some strains, the sulfate esters constituent of the extracellular polysaccharide sheaths.

Regulation

The regulation of cellular activities in cyanobacteria is similar in nature to that found in other prokaryotes, but photobiology plays a particularly important role. The presence and nature of the cyanobacterial photoreceptor systems (a cell's light meter) are well documented. Although specific photoreceptor molecules, some structurally similar to plant phytochromes, do exist, and some have been postulated as sensors of UV radiation, in many cases it is the indirect effect of light supply on the overall redox state of the cell that determines cellular responses. Small redox-sensitive proteins such as thioredoxin may act as general modulators of enzyme activity in carbon and nitrogen metabolism. The balance between carbon and nitrogen metabolism is typically sensed through the levels of 2-oxoglutarate. Short-term (photo)responses can also be based on protein phosphorylation mechanisms, as seems to be the case for the process leading to the redistribution of captured energy between photosystems I and II (the so-called state transitions) or for the direct regulation of phosphoenolpyruvate carboxylase activities. By means of its multiple targeting, phosphorylation of a serine residue of P_{II}, a small regulatory protein, is thought to provide coordinated regulation of carbon and nitrogen metabolism. There is abundant evidence for light-mediated regulation of gene expression, leading to long-term responses, either to light intensity or to spectral composition. This is particularly true for genes encoding components of the photosynthetic apparatus, such as phycobiliproteins and PSII polypeptides. Some strains grown under light–dark cycles are capable of incorporating specific metabolic tasks into the swing of the cycle, relegating, for example, protein synthesis or nitrogen fixation to the dark periods. At least some of these daily patterns are maintained by an internal clock system, because the periodicity remains even in the absence of environmental stimuli. The core of this central clock, virtually universal among cyanobacteria, is an autophosphorylating enzyme, KaiC, that oscillates between nonphosphorylated and phosphorylated forms, and can modulate the expression of various central genes. Two-component (histidine kinase/response regulator) regulators seem to be common for signal transduction, particularly in responses to environmental stress or shock (cold, heat, salt, and light), as well as for adaptation to some forms of nutritional limitation. Regulatory, noncoding RNAs are also known for a few genes.

Motility and Taxes

Cyanobacteria do not have flagella, but many unicellular and filamentous cyanobacteria display gliding motility. In some strains of oceanic marine *Synechococcus*, slow-swimming motility has also been described. Gliding is a movement across a solid or semisolid material in the absence of flagella or other conspicuous propulsion mechanisms and without apparent change in cellular (or trichome) shape. Gliding is typically accompanied by the secretion of slime. In filamentous forms, rotation of the trichomes along their main axes often occurs while gliding. The structural involvement of a Ca^+-binding glycoprotein, oscillin, in cyanobacterial gliding has been determined; it forms supracellular helical fibrils in the outermost surface of the trichomes. However, the actual mechanism of cyanobacterial motility remains unknown, and may simply not be universal among them. Gliding motility may be displayed only transiently (i.e., in hormogonia or in baeocytes but not in vegetative cells). Photosensory and chemosensory systems, allowing the organisms to respond to temporal or spatial environmental gradients, are tightly coupled to motility, resulting in the so-called tactic behavior. Positive tactic responses to chemical species (chemotaxis) such as bicarbonate and nitrate have been shown in cyanobacteria (i.e., they move up chemical gradients of concentration of those substances). Terrestrial cyanobacteria are the only microorganisms shown to have tactic responses to water. All motile cyanobacteria display phototactic behavior so that the populations are able to seek optimally illuminated areas. Like other bacteria, cyanobacteria usually respond by stopping and changing the direction of movement (reversing) upon crossing a sharp boundary in light intensity (photophobic response); however, some are also capable of perceiving the angular direction of the light and responding by steering toward or away from the direction of the incoming light. This capacity (known as true phototaxis) has no parallel in any other prokaryote.

Molecular Genetics

Genomes

The genome is typically prokaryotic in nature and is located in the centroplasm. The genomes of free-living cyanobacteria vary widely in GC base composition from 32 to 71%, a range comparable to that spanned by all bacteria. They also vary in size, approximately correlating with morphological complexity, from 1.6 to 14×10^6 bp (base pairs) (~1700–10 000 genes). The smallest cyanobacterial genomes are thus similar in size to those of most bacteria, whereas the largest ones are in the range of eukaryotic fungal genomes. Symbiotic cyanelles and plastids have retained only 0.13×10^6 bp in their genomes. To date, more than 30 cyanobacterial genomes have been fully sequenced. The DNA of cyanobacteria is subject to very extensive modification, which in some cases is so thorough that a role for methylation beyond protection from restriction enzyme cleavage has been postulated. The presence of widespread, highly iterated short palindromic sequences is a trait shared by many, but not all, cyanobacterial genomes. Genomic rearrangements involving deletion, operon fusion, and translocation events are known to occur during heterocyst differentiation. Plasmids or extrachromosomal replicons are commonly encountered (some as large as 1.5×10^5 bp), but they are usually cryptic and do not appear to be responsible for antibiotic resistance phenotypes, as in other bacteria. Some are known to bear genes encoding for isozymes involved in assimilatory sulfate reduction.

Gene Transfer

There is evidence from phylogenetic comparisons that horizontal genetic exchange among related cyanobacteria has played a significant role in their evolution. Nevertheless, the mechanisms leading to genetic exchange are difficult to pinpoint. Despite the abundance and spread of cyanobacterial plasmids, natural conjugation among cyanobacteria has not been reported. The same is true for viral transduction, despite the wealth of cyanophages described in the laboratory and from natural populations, some clearly carrying cyanobacterial genes in their genomes. Some strains are naturally highly competent for taking up foreign DNA, but unaided transformation seems to be restricted to some unicellular strains of the genera *Synechococcus* and *Synechocystis*.

Gene Expression

Control of gene expression at the level of transcription seems to play a significant role in the adaptation to changing environmental conditions. Cyanobacteria possess a transcriptional apparatus of unique characteristics among bacteria. The cyanobacterial DNA-dependent RNA polymerase is structurally different from that of the common bacterial type, possessing an additional subunit in its core, and several sigma factors (polypeptides, whose association with the core of the polymerase is needed for effective initiation of transcription) have been identified. It has been shown that a ‘principal’ sigma factor is commonly present under normal growth conditions, whereas alternative factors are temporarily expressed upon, for example, a change to nitrogen-limiting conditions. Because cyanobacterial promoters lack some of the distal consensus sequences of other bacteria, it has been hypothesized that regulation of transcription may often be activated by accessory factors other than sigma factors. Noncoding RNA transcriptional regulatory elements are known for a few genetic systems, such as the fur (ferric uptake regulator) master regulator and the *isi*A locus, both related to iron limitation, and may be more widespread than presently considered.

Ecology and Adaptations

The range of environmental conditions under which cyanobacteria can develop is impressively wide, and equally wide is the variety of ecological adaptations they display. One can find cyanobacteria as an important part of the primary producer community in almost any habitat in which light penetrates. Thermophilic cyanobacteria can grow up to temperatures of 73 °C in hot springs, which is the upper temperature limit for any phototrophic organism, and develop stable populations in polar soils, rocks, and ponds in which temperatures rarely exceed a few degrees Celsius. Some forms thrive in rain or snow-melt puddles of extremely low inorganic solute concentrations, and some halotolerant types grow in NaCl-saturated brines. Cyanobacteria thrive in caves, deep in lakes, and in coastal areas, where light is extremely dim, but some terrestrial forms develop permanent populations in mountainous tropical areas exposed to the highest levels of solar radiation found on Earth. Many terrestrial cyanobacteria are desiccation resistant, and they withstand freeze–thaw cycles. Benthic marine cyanobacteria flourish under supersaturated oxygen, often exceeding 1 atm in partial pressure during daytime, but they are exposed to anoxia at night. It is common that more than one of these extreme conditions coincide in one particular habitat. One of the most conspicuous limitations to the development of cyanobacteria seems to be acidity: although many are known from alkali lakes, no *bona fide* reports of growth below pH 4.5 exist. The ecological success of cyanobacteria in many of these extreme habitats is often a result of their metabolic resilience in the face of environmental insults rather than a consequence of sustained growth. A few environmentally relevant cyanobacterial habitats are discussed in the following sections.

Marine Plankton

With the possible exception of polar areas, morphologically simple, cyanobacteria of small size (0.5–2 μm) inhabit in large numbers in the upper zone of the oceans where light penetrates. These are referred to as picoplankton, and consist of two phenotypically distinct but phylogenetically related groups (**Figure 2**): the open-ocean marine *Synechococcus* and *Prochlorococcus*. Population sizes typically range between 10^4 and 10^5 cells per ml for both types. The global biomass of picoplankton must be on the order of 1–2 billion metric tons (1600×10^{12} g). This shear size indicates their ecological importance. It has been calculated that as little as 11% and as much as 50% of the primary production of nonpolar open ocean regions is due to their activity. This group has developed interesting adaptations to the light field of clear oligotrophic waters: their light-harvesting complexes have differentiated to match the predominantly blue light available. *Synechococcus* cells synthesize a special kind of bilin chromophore, phycourobilin, absorbing maximally at 490–500 nm, thus increasing the ability of cells to use blue light. Evolutionary pressure of a similar nature has probably resulted in the virtual loss of phycobiliprotein-based light harvesting in *Prochlorococcus* and the evolution of antenna mechanisms based on (divinyl) chlorophylls (*a* and *b*), which are optimally suited to capture blue light. The life strategy of picoplankton populations is based on fast growth, with cells often displaying several doublings per day. Grazing pressure and viral infection seem to be the major factors controlling population sizes. The comparatively small size of picoplankton genomes, the absence of nitrogen-fixing capacity and of some reserve polymers such as phycocyanin, and the lack of mechanisms to withstand small concentrations of toxic metals such as copper may be the result of reductionist evolutionary pressures favoring fast growth. Their small size (large surface to volume ratio) may provide selective advantage in nutrient-poor environments. Phenotypic and genetic variation exists within the picoplanktonic cyanobacteria, resulting in strains that diverge in light and temperature optima for growth: high- and low-light-loving strains of *Prochlorococcus* have been described as mesophilic and moderately psychrophilic strains of *Synechococcus*.

The intensely red (phycoerythrin-containing) oscillatorian cyanobacteria of the genus *Trichodesmium*, which typically occur as bundles of filaments in the wild, constitute the second most important group of marine planktonic cyanobacteria, with a global biomass estimated at 100×10^{12} g. They are inhabitants of oligotrophic (nutrient-poor) tropical open ocean regions worldwide, in which they may form blooms that can be detected as surface accumulations with the naked eye. They are responsible for much of the global oceanic nitrogen fixation, and this nitrogen-fixing capacity is a key factor of their ecological success. The particular adaptations that allow nonheterocystous *Trichodesmium* filaments to fix nitrogen in the light, however, are not well understood. They also contain large amounts of gas vesicles that provide positive buoyancy to the filaments so that they remain in the upper wind-mixed layers of the ocean. *Trichodesmium* gas vesicles are among the sturdiest in prokaryotes, apparently so that they can withstand the large hydrostatic pressures experienced upon mixing of the deep mixed layers of open-ocean waters. Large, unicellular populations of '*Crocosphaera*' also contribute significantly to the inputs of new nitrogen in the oceans along with *Trichodesmium.*

Freshwater Plankton

Although not a major component of the global biomass (with only some 30×10^{12} g), a large variety of cyanobacteria are found as components of the phytoplankton of fresh waters, and are particularly prominent or dominant under conditions of nutrient eutrophication. In eutrophic lakes and man-made reservoirs (and in enclosed brackish water basins such as the Baltic), the formation of cyanobacterial blooms results in serious water quality problems regarding not only the degradation of the recreation potential, musty odors, and off-flavors that are associated with bloom development, but also the likelihood of fish kills due to anoxic events after bloom decay and the production and release of cyanobacterial toxins. These are known to have caused animal and, in extreme cases, human deaths. Bloom-associated health effects reach beyond local environmental agencies and are being considered in rulings of the World Health Organization. Gas-vacuolated species in the heterocystous genera *Anabaena*, *Nodularia*, '*Gloeotrichia*', and *Aphanizomenon*, as well as in the nonheterocystous genera *Oscillatoria* and particularly *Microcystis*, are notoriously responsible for bloom formation and for reported cases of intoxication.

Terrestrial Environments

Desiccation-resistant terrestrial cyanobacteria have widespread occurrence. They may be found growing on bare surfaces (rocks, trees, buildings, and soils) or several millimeters within more or less soft diaphanous substrates (soils, sandstone, and limestone). Some species actively bore into the rock substrate. The availability of liquid water, in the form of rain or dew, determines the potential spurts of growth of cyanobacteria in the terrestrial environment. Growth of terrestrial cyanobacteria can be fast and luxurious in tropical humid climates, but in most other regions it is usually only intermittent. Their life strategy is usually one of slow growth and enhanced resilience. Adaptations to this environment are directed to withstand both desiccation (e.g., by abundant exopolysaccharide production) and exposure to solar radiation under inactive conditions (by the synthesis of sunscreen pigments). The exclusion of higher plant vegetation by climatic rigors determines the relative importance of cyanobacteria in terrestrial habitats. Thus, extensive endolithic cyanobacterial communities (accounting for as much as 140×10^{12} g of biomass globally), usually dominated by members of the genus *Chroococcidiopsis*, have been described from tropical, desert, and polar environments. These communities play a significant role in rock erosion processes, and their actions have become a concern for the preservation of stone monuments. Edaphic (soil dwelling) cyanobacteria are also distributed worldwide, and represent one of the largest global reservoirs of biomass, with some 540×10^{12} g. Sheathed oscillatorian forms such as *M.*'*vaginatus*', possibly the most common and widespread, along with heterocystous ones (*Nostoc* and *Scytonema*), are major ecological players in arid and semiarid regions, both hot and cold. Edaphic cyanobacteria in the so-called biological soil crusts contribute significantly to the physical stability and fertility of arid soils worldwide.

Sulfidogenic Environments

Hydrogen sulfide interferes with PSII and acts as a potent inhibitor of oxygenic photosynthesis. Many marine and fresh-water habitats, such as hot springs, marine littoral sediments, and the deep water of lakes, may contain significant amounts of free sulfide. Cyanobacteria develop the most conspicuous populations of oxygenic phototrophs in such environments when sufficient light is available. Specific adaptations to these habitats include the ability to express sulfide-resistant forms of PSII so that oxygenic photosynthesis can proceed even in the presence of sulfide (e.g., in the marine benthic *Microcoleus* '*chthonoplastes*' and in some hot spring and freshwater oscillatorians) and also an ability to perform anoxygenic photosynthesis using hydrogen sulfide as a source of electrons instead of water (e.g., in *Oscillatoria* '*limnetica*' and members of the 'Halothece' cluster from hypersaline waters, *Oscillatoria* '*amphigranulata*' from hot springs, or *Pseudanabaena* sp. from hardwater lakes). Many strains display both adaptations simultaneously. The ability to use sulfide as an electron donor has been traced to the inducible expression of a soluble enzyme, sulfide:quinone oxidoreductase, which can transfer electrons from sulfide to plastoquinone, thus allowing the noncycling function of PSI (**Figure 5**) with the formation of both ATP and NADPH. Although some strains in culture show continued growth using anoxygenic photosynthesis alone, they cannot compete successfully for sulfide with phototrophic sulfur bacteria in the environment. It is thought that cyanobacteria use anoxygenic photosynthesis as a means for sulfide detoxification. Indeed, many use anoxygenic photosynthesis only temporarily, until local concentrations of sulfide are sufficiently low and (sulfide-resistant) oxygenic photosynthesis can begin.

Symbioses

Although they show an apparent lack of taste for sexual matters, cyanobacteria have displayed a considerable evolutionary promiscuity, entering into intimate symbiotic associations with various unrelated organisms. The list of cyanobacterial symbioses is large. There is also a large variation in the degree of independence maintained by the cyanobacterial partners. In some cases, the distinction of two organisms may no longer be possible because cyanobacteria lose their typical appearance and a large portion of their genomes to their hosts. This is obviously the case in higher plant and eukaryotic algal plastids, in which massive loss of genes to the eukaryotic nucleus has occurred. Cyanelles (plastid-like endosymbionts of eukaryotic protists) have retained the peptidoglycan and phycobilines, but their identity loss is substantial as well. These cases are no longer considered symbioses. At the other end of the spectrum, loose but mutualistic relationships between cyanobacteria and other bacteria or fungi (the so-called consortia) have been described, but these are not considered here. Well-known cyanobacterial symbioses can be functionally divided into those formed by heterocystous cyanobacteria, in which the main contribution of the cyanobacterial partner is the supply of fixed nitrogen, and those formed by nonheterocystous types, in which their contribution is often the supply of fixed carbon. According to the degree of intimacy attained, they can be classified into intracellular (in which cyanobacterial cells are found within cells of other organisms) and extracellular (in which cyanobacterial cells are located within the tissues but outside the cells of other organisms). The most common extracellular symbioses of nonheterocystous cyanobacteria (involving the unicellular genera *Chroococcidiopsis*, *Gloeocapsa*, '*Chroococcus*', and *Gloeothece*) are in the form of cyanolichens. Both *Prochloron* and large-celled *Synechocystis* are known from extracellular symbioses with ascidians in tropical or subtropical marine waters. Extracellular symbioses of Pseudanabaena-like '*Konvophoron*' occur in Mediterranean sponges. Filamentous *Phormidium* has been reported in symbioses with some green algae. Intracellular symbioses of nonheterocystous cyanobacteria are known from tropical sponges ('*Aphanocapsa*', *Oscillatoria*, *Synechocystis*, and *Prochloron*), from green algae (*Phormidium*), and from dinoflagellates (unidentified). Heterocystous cyanobacteria in the genus *Nostoc* are known to form extracellular symbioses with liverworts and higher plants (Cycads and duckweed). *Anabaena* enters into symbiosis with water ferns of the genus *Azolla*. Cyanolichens are known to contain members of the genera *Nostoc*, *Calothrix*, *Scytonema*, *Stigonema*, and *Fischerella* as photobionts. Intracellular symbioses of heterocystous cyanobacteria occur in oceanic diatoms of the genera *Hemiaulus* and *Rhizosolenia* (cyanobacterial genus '*Richelia*') and in *Trifolium* (clover) with *Nostoc*. *Nostoc* also enters into intracellular symbioses with the terrestrial nonlichenic fungus *Geosiphon pyriforme*.

Fossil Record and Evolutionary History

The fossil record of cyanobacteria contains the oldest entries that can be confidently assigned to any extant group of organisms. Excellently preserved microfossils, 1000 million years old, bear virtually indisputable cyanobacterial morphologies. Fossil cyanobacteria showing considerable morphological diversification have been described dating back at least 2500 million years. Additionally, filamentous bacteria of putative cyanobacteria identity are known from as far back as 3500 million years. In fact, it has been suggested that cyanobacteria have evolved only very slowly in the intervening time because present and past morphologies are very similar. In view of the biochemical and physiological diversity of adaptations that particular cyanobacteria display, some doubts may be cast on such a perception. The fossil record of the Archaean and Proterozoic Eons (before 500 million years ago) offers strong evidence not only for the presence of cyanobacteria but also for a type of environment they must have inhabited: the sedimentary environment of shallow coastal waters. This is recorded in the abundant organosedimentary laminated macrofossils known as stromatolites, which became geographically restricted in the Phanerozoic. Stromatolites are analogous to present-day cyanobacterial mats, benthic compact assemblages built by cyanobacteria in extreme environments, and have provided evidence for the sustained importance of photoresponses in the ecology of cyanobacteria in the form of 'heliotropic' accretions. Precambrian fossil microboring on marine carbonaceous substrates reveals the sustained role of cyanobacteria in small-scale geomorphological processes. Fossil evidence for the presence of eukaryotic algae is also quite old, perhaps as much as 2000 million years, which is in agreement with the early offshoot of the plastidic line of evolution suggested by phylogenetic reconstructions. The oldest fossil evidence for terrestrial cyanobacteria, in the form of *Gloeocapsa*-like cells symbiont in lichens, is comparatively young (400 million years). Thus, cyanobacteria have inhabited Earth for a long time and survived through geological periods of environmental conditions very different from those reigning today. In the early days of cyanobacterial evolution, high fluxes of short-wavelength UV radiation penetrated the oxygen- and ozone-free, carbon dioxide-rich atmosphere. Oceans were shallow and rich in reduced iron and poor in sulfate and nitrate. In fact, it is thought that oxygenic photosynthesis was the ultimate cause, regulated by geological events of carbon burial, for the change in most of these parameters, including the late Proterozoic oxygenation of the atmosphere.

Comparative biochemistry of the proteins in the different photosystems suggests homologies between PSII of cyanobacteria and the photosystems of purple sulfur bacteria, as well as between PSI and the photosystems of green sulfur bacteria. The hypothesis has been presented that genetic fusion between different oxygenic phototrophs may have led to the evolution of a two-photosystem photosynthetic apparatus in the predecessor of cyanobacteria, perhaps using iron as an electron donor. The evolutionary lowering of the basal potential of the type II photosystem, allowing the retrieval of electrons directly from water, would have supposed the tapping of a virtually unlimited and ubiquitous source of electrons for CO_2 reduction, providing the first cyanobacterium with a wide range of potential niches and possibly enabling an early explosive radiation of particular adaptations.

Commercial Use and Applications

The commercial use of cyanobacteria has long been sought for but in most cases has not reached the production stage. Procedures and modified strains have been devised, for example, for the industrial production of amino acids, ammonia, and for the control of mosquito larvae using genetically engineered strains that produce *Bacillus* toxins. The main commercial use of cyanobacteria is for the production of bulk biomass for human consumption, a practice that has a long history in traditional cultures. Natural blooms of *Arthrospira* (previously assigned to *Spirulina*) were collected, sun-dried, and cut into cakes for human consumption in preHispanic Mexico; this 'tecuitlatl' of the Aztecs was highly regarded and commercialized at that time. A very similar procedure is used today to manufacture 'Dihé' cakes by the Kanembu tribeswomen from the shores of Lake Chad. Indeed, dried *Arthrospira* contains 60–70% protein. Today, it is also produced commercially in outdoor man-made facilities and commercialized under the trade name 'Spirulina' for the health-food market as a protein-rich, low-calorie, cholesterol-free, vitamin-loaded food supplement. Due to the cult that has developed regarding this form of food supplement, blooms of other species ('Aphanizomenon', traditionally not consumed and strains of which are known to contain toxins) are also being commercially sold. *Nostoc commune*, a terrestrial cyanobacterium, is considered a delicacy and has been collected and marketed for centuries in China. Given the central role that natural populations of cyanobacteria play in maintaining the long-term fertility of paddy soils for rice cultivation, inoculating rice fields with cyanobacterial mixtures is currently a standard agricultural practice in some Asian countries. The symbiotic association *Anabaena/Azolla* (see 'symbioses') is intensively cultivated in the Far East for its use as green manure and as fodder for poultry and swine; written instructions for this practice date to 500 BC. In addition, on a much smaller scale, cyanobacteria are used as sources for fine biochemicals. Beta-carotene and phycocyanin are commercialized as food colorants. Chlorophyll *a*, radiolabeled nucleotides, and amino acids and some restriction endonucleases of cyanobacterial origin are sold for research purposes. The possibility of using cyanobacteria biomass biofuels, as suppliers of biomass, biodiesel, or hydrogen, is currently being pursued by a variety of academic and industrial ventures worldwide.

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Deep-Sea Hydrothermal Vents

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Defining Statement
Mid-Ocean Ridge Hydrothermal Vents
The Chemosynthetic Basis of Life at Hydrothermal Vents
Chemolithoautotrophic Bacteria
Heterotrophic Vent Bacteria
Hyperthermophilic Archaea
Comparison to Terrestrial Hot Springs
Unusual Hydrothermal Vents: Loihi, Guaymas, Lost City
Further Reading

Glossary

archaea One of the two prokaryotic domains of life, phylogenetically and also biochemically distinct from the bacteria. The metabolic and physiological properties of archaea from hydrothermal vents reflect adaptations to challenging environmental regimes; vent archaea include anaerobic and microaerophilic thermophiles and hyperthermophiles, methanogens and anaerobic methane oxidizers, heterotrophic and autotrophic archaea.

black smoker A common type of hydrothermal vent where extremely hot (~350 °C) vent fluid emerges into the water column through channelized flow in a chimney-like structure; the chimneys are formed by precipitation of metal sulfides from the vent fluid and can grow to a height of less than 1 to more than 10 m, depending on the stability of precipitated minerals. The porous chimney matrix is the major natural habitat of hyperthermophilic vent archaea.

chemolithoautotroph A microorganism that obtains its metabolic energy from the oxidation of reduced inorganic compounds, and its carbon for biosynthesis from CO_2 or CO.

chemosynthesis The use of chemical energy instead of light to drive the assimilation of CO_2 as the sole carbon source for biosynthesis of cellular material; chemical energy comes from light-independent oxidation of inorganic electron donors.

electron acceptor The oxidant (e.g., O_2, NO_3^-, SO_4^{2-}, Fe^{3+}, CO_2) in a biologically mediated redox reaction that accepts electrons from the reductant or electron donor, an organic compound, or an inorganic electron donor (H_2S, H_2, reduced metals, CH_4). The combination and concentrations of oxidant and reductant determine the energy yield of a microbial redox reaction.

electron donor At hydrothermal vents, inorganic electron donors in the vent fluid (H_2S, H_2, reduced metals, CH_4) are used as the primary electron donors and energy sources for microbial metabolism by chemolithotrophic bacteria and archaea. Organic carbon compounds from pyrolyzed cells serve as electron donors for heterotrophic bacteria and archaea.

heterotroph A microorganism that requires organic carbon as energy and carbon source. At hydrothermal vents, heterotrophs degrade biomass and organic substrates derived from chemolithoautotrophic bacteria and archaea; in the surface biosphere, heterotrophs rely on photosynthetic biomass.

hyperthermophile An extremely thermophilic bacterium or archaeon with an optimum growth temperature above 80 °C.

mid-ocean ridge The region where two opposite oceanic plates are separating a few centimeters every year; the split (the spreading center) is characterized by increased volcanic and tectonic activity and hydrothermal venting. This region is elevated 2–3 km above the deep-sea floor (4–5 km average depth) and appears as an extensive underwater mountain chain or ridge.

Abbreviations

ANME	anaerobic methane-oxidizing
RuBisCO	ribulose-1,5-bisphosphate carboxylase–oxygenase

Defining Statement

Hydrothermal vents, hot seafloor springs at mid-ocean ridges, sustain chemosynthetic microbial ecosystems that are independent of photosynthetically produced biomass; these hot, acidic, and chemically toxic habitats harbor a wide, only incompletely recognized spectrum of extremophilic bacteria and archaea that are specifically adapted to the hydrothermal vent environment.

Mid-Ocean Ridge Hydrothermal Vents

Hydrothermal vents are hot deep-sea springs at mid-ocean ridge spreading centers where the extensive basalt plates that form the basement of the seafloor (oceanic crust) are split apart and new ocean crust is constantly generated from melting zones in the earth's upper mantle. They occur globally and follow the mid-ocean ridges and their spreading centers that meander around the ocean surface of the earth, almost as seams around a tennis ball. At slow spreading centers such as the Mid-Atlantic Ridge, the area between the separating oceanic plates just above the geothermally active spreading zone appears as a valley or trough (the 'Rift Valley') framed by the higher ridges of the young basaltic crust on both sides of the spreading center; fast-spreading centers such as the East Pacific Rise show often an 'axial high', an upward bulge sandwiched between the separating plates. These areas at the hot, volcanically active center of the mid-ocean ridges are the location of most hydrothermal vents; fewer, geologically and chemically distinct types of vents occur at some distance from the mid-ocean ridge axis. Most hydrothermal vents occur at water depths between 2000 and 3500 m, corresponding to the water depth of most mid-ocean ridges.

The fractured and porous basement rock of the mid-ocean ridge entrains cold deep-sea water, which then circulates through the subsurface underneath the spreading center, analogous to water circulating through an aquifer on land. During subsurface passage, seawater is exposed to the geothermal heat of the melting zone underneath the spreading center; the chemistry of the entrained seawater is fundamentally altered as it reacts with the subsurface basalt and undergoes phase separation under extreme temperature and pressure. The most significant and microbiologically relevant changes include sulfate removal by anhydrite precipitation ($CaSO_4$) and by geothermal reduction of seawater sulfate to hydrogen sulfide; leaching of metals and of additional sulfur from the subsurface basalt; generation of protons and decreasing pH toward 3–4 during water–rock interaction; removal of oxygen due to outgassing; increasing CO_2 concentration as a consequence of magma degassing; and elevated hydrogen and methane concentrations. In most cases, H_2S and CO_2 are the dominant component of vent fluids, with concentrations between 3 and $>100\,\text{mmol}\,l^{-1}$. The highly altered hydrothermal vent fluids reach temperatures of 300–400 °C, but remain in the liquid state due to the high hydrostatic pressure of the deep sea. The hot, buoyant hydrothermal fluid migrates back to the seafloor surface and emerges as a high-temperature vent; alternatively, subsurface seawater mixing can attenuate the temperature and chemistry, resulting in warm vents (**Figure 1**). High-temperature vent fluids appear as a black or gray cloud, undergoing instant precipitation of dissolved metal sulfides as the vent fluid mixes into the cold, oxygenated deep-sea water column. The precipitated minerals, generally metal sulfides and anhydrite, accumulate *in situ* and build a friable and porous structure that surrounds the hot fluid flow, channels and focuses it; the result is the hydrothermal chimneys from which the fluid flow emerges (**Figure 2**). These porous and friable structures can grow to tens of meters in height, depending on local chemistry and the stability of the precipitated minerals. Their walls are only a few centimeters thick, but separate the channelized flow of the hydrothermal vent fluid (300–400°C) within the chimney from the cold, oxygenated deep-sea water surrounding the chimney and cooling its surface (~2 °C). The steep temperature and chemical gradients within the chimney walls are the preferred habitat for anaerobic, hyperthermophilic vent archaea (**Figure 3**) and provide the source material for enrichment and isolation of the most thermophilic microorganisms on Earth.

Mixing of hydrothermal fluids with entrained seawater within the porous subsurface of a hydrothermal vent site produces a wide range of warm vent fluids, which emerge as channelized or diffuse flow (**Figure 1**). The moderate temperatures and the changes in chemistry favor different microbial populations. Hyperthermophilic archaea originating in the hot subsurface and entrained in the mixed fluids remain detectable, but the dominant populations that are sustained by these mixed vent fluids are sulfur-oxidizing autotrophic bacteria with a mesophilic or moderately thermophilic temperature optimum. These bacteria constitute the dominant primary producers of biomass at hydrothermal vents and fall into two broad classes: free-living, surface-attached or mat-forming bacteria and bacterial symbionts of marine invertebrates that contribute to the nutrition of their hosts.

Hydrothermal vents are subject to sudden geological disturbance by earthquakes and volcanic eruptions that occur frequently at mid-ocean ridges; in addition, the precipitation of minerals, such as metal sulfides, on the subsurface can alter the deep plumbing of a hydrothermal vent system. Thus, individual hydrothermal vent sites have short life spans, in the range of a few years or decades; the microbial communities

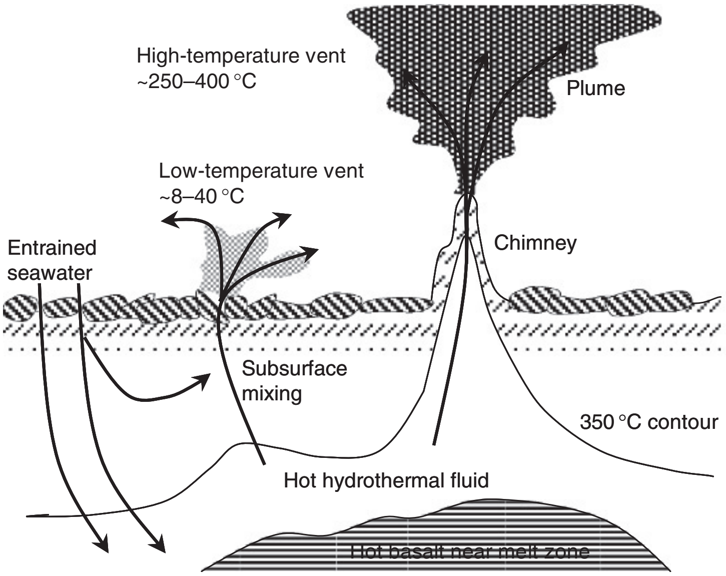
High-temperature vent
~250–400 °C
Plume
Low-temperature vent
~8–40 °C
Entrained seawater
Chimney
Subsurface mixing
350 °C contour
Hot hydrothermal fluid
Hot basalt near melt zone

Figure 1 Schematic cross-section of hydrothermal circulation at a mid-ocean ridge. Seawater is drawn into the crust, heated to 350–400 °C, chemically altered by interaction with the hot basalt subsurface, and rises buoyantly back to the seafloor. At the seafloor, venting high temperature end member fluids precipitate chimneys; subsurface mixing with seawater produces lower temperature vents. Modified after McCollom TM and Shock EL (1997) Geochemical constraints on chemolithoautotrophic metabolism by microorganisms in seafloor hydrothermal systems. *Geochimica et Cosmochimica Acta* 61: 4375–4391.

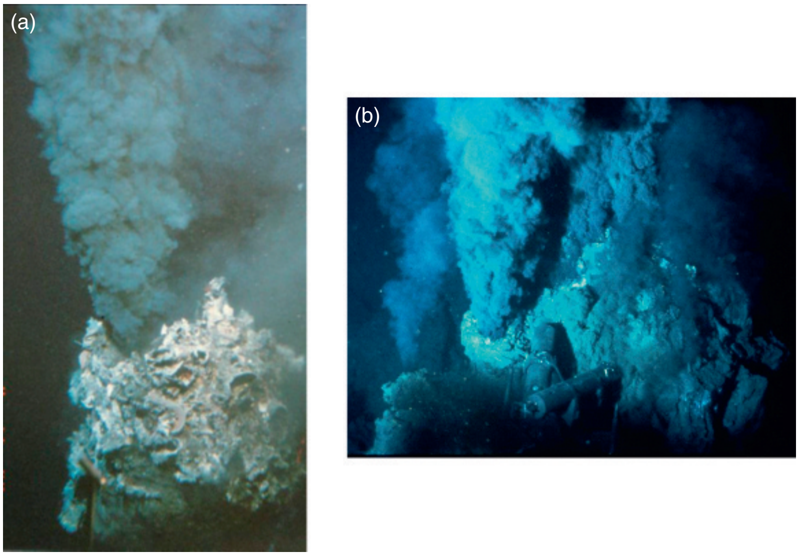

Figure 2 (a). Black smoker chimney on the East Pacific Rise (21°N) at 2600 m depth. The orifice of the chimney consists of whitish aragonite ($CaSO_4$); the lower chimney consists of the gray-black metal sulfides. (b). A group of black smoker chimneys at East Pacific Rise (21°N). Photo credits: Jannasch H, WHOI.

and the symbiont-dependent vent animals recolonize new vents quickly as previous vent habitat may disappear at short notice. The microbial communities of hydrothermal vents are resilient and have maintained themselves in their volatile and extreme habitat for billions of years.

The Chemosynthetic Basis of Life at Hydrothermal Vents

The hydrothermal vent ecosystem is based on chemolithoautrophic bacteria and archaea that derive energy from the oxidation of inorganic compounds, mostly

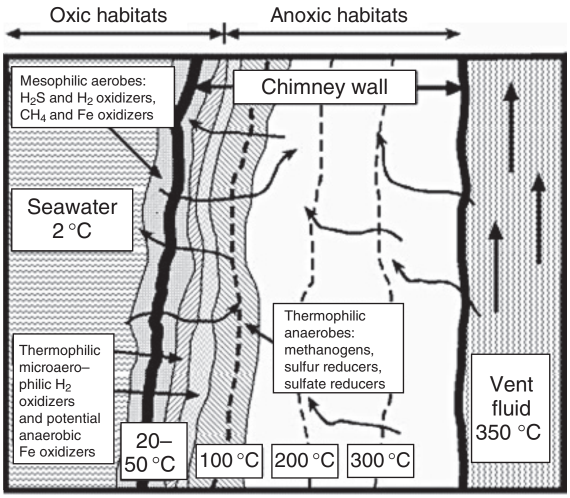
Oxic habitats
Anoxic habitats
Mesophilic aerobes: H_2S and H_2 oxidizers, CH_4 and Fe oxidizers
Chimney wall
Seawater 2 °C
Thermophilic anaerobes: methanogens, sulfur reducers, sulfate reducers
Thermophilic microaero–philic H_2 oxidizers and potential anaerobic Fe oxidizers
Vent fluid 350 °C
20–50 °C
100 °C
200 °C
300 °C

Figure 3 Schematic cross-section of a hydrothermal vent chimney wall, showing habitats for thermophilic and mesophilic bacteria and archaea. Anaerobic archaea are found around the 100 °C isothermes and possibly deeper within the chimney wall. Thermophilic, microaerophilic hydrogen-oxidizing bacteria (Aquificales) occur further toward the outer chimney layers where dissolved oxygen penetrates in low concentrations. Mesophilic, aerobic hydrogen-, sulfur- or methane-oxidizing bacteria grow on the chimney surface. Modified after McCollom TM and Shock EL (1997) Geochemical constraints on chemolithoautotrophic metabolism by microorganisms in seafloor hydrothermal systems. *Geochimica et Cosmochimica Acta* 61: 4375–4391.

sulfide or hydrogen (lithotrophy), and build up their biomass by assimilation of dissolved inorganic carbon, such as CO_2, CO, HCO_3^-, or CO_3^{2-} (autotrophy). The oxidation reactions of the inorganic electron donors are exergonic and proceed without additional energy input. The energy thus obtained drives proton transport across the cytoplasmic membrane and is conserved as ATP. Also, it drives reverse electron transport and the reduction of physiological hydrogen carriers; these are, together with ATP, essential for autotrophic carbon fixation. The shortened term 'chemosynthesis' is generally used for this mode of microbial life, in analogy to photosynthesis, where the oxidation of an inorganic electron donor requires the input of light energy.

Depending on the preferred electron acceptor, chemosynthetic metabolism can be aerobic or anaerobic. Oxidation reactions with oxygen or nitrate as terminal electron acceptor give the highest energy yields, which explains the great diversity and abundance of oxygen- or nitrate-dependent chemolithotrophic bacteria at hydrothermal vents (see 'Free-living chemolithoautrophic bacteria'). Oxygen-respiring chemolithoautotrophs have also evolved numerous symbiotic associations with marine invertebrates, where the animal host optimizes the supply of electron donor and acceptor in return for nutritional contributions from its interior or exterior symbiont (see 'Symbiotic chemolithoautotrophic bacteria'). Chemolithoautotrophic metabolism is also possible with electron acceptors other than oxygen, such as oxidized metals, oxidized sulfur species, and inorganic carbon in the oxidation state of CO_2, CO, and formate. Since chemosynthesis cannot take place without oxidized electron acceptors, the hydrothermal vent ecosystem remains geochemically linked to the photosynthetic biosphere near the ocean surface. Only photosynthesis produces oxygen, which in turn is essential for producing other oxidized electron acceptors. For this reason, hydrothermal vent ecosystems are not independent domains of life that could survive and thrive even if some planetary catastrophe extinguishes photosynthetic surface life. The only possible exceptions are chemolithoautotrophic methanogenic archaea, which generate methane from CO_2 and hydrogen and use reactions of the acetyl-CoA pathway for autotrophic carbon fixation. CO_2 is abundant in hydrothermal fluids and could, together with hydrogen of hydrothermal origin, sustain an autotrophic, autonomous hydrothermal vent biosphere. For the same reason, CO_2/H_2 autotrophic methanogenesis is a good candidate for a microbial pathway that can sustain autonomous life in the deep subsurface, for example, in deep marine sediments or the basaltic ocean crust.

The *in situ* chemical conditions determine which autotrophic pathway or life strategy contributes most to the overall biomass at hydrothermal vents. Where sulfide and oxygen coexist in turbulent mixing of vent water and seawater, symbiotic chemosynthetic bacteria (sulfur-oxidizing, oxygen-respiring chemolithoautotrophs) and their animal hosts make the highest contribution of new organic carbon to hydrothermal vent ecosystems; the symbiotic bacteria sustain not just their own biomass, but their host animals as well. In some cases, symbiont-harboring invertebrates reach unusually large sizes and body mass. The vent clam *Calyptogena magnifica* can reach a length of a foot and weigh over a pound; the vestimentiferan vent worm *Riftia pachyptila* reaches a length of 2 m, two orders of magnitude larger than its closest, nonhydrothermal relatives. Nonsymbiotic, free-living sulfur-oxidizing bacteria can also grow in an abundance that is not seen anywhere else; for example, the mat-forming filamentous bacterium *Beggiatoa*, which oxidizes sulfide and elemental sulfur with nitrate as electron acceptor, grows in thick pillows in suitable locations (see 'Unusual hydrothermal vents: Loihi, Guaymas, Lost City').

The dominant microbial pathway of autotrophic carbon fixation at hydrothermal vents was assumed to be the Calvin–Benson–Bassham cycle, found in numerous free-living and symbiotic sulfur oxidizers that are phylogenetically related to each other, as members of the γ-Proteobacteria. However, recently discovered and phylogenetically distinct bacterial populations (sulfur-and hydrogen-oxidizing bacteria within the ε-Proteobacteria) were using the reverse TCA cycle for carbon fixation; this pathway also exists in symbiotic sulfur oxidizers.

Chemolithoautotrophic Bacteria

Free-Living Chemolithoautrophic Bacteria

Most free-living, nonsymbiotic autotrophs in the hydrothermal vent environment belong to three major physiologically and phylogenetically distinct groups of sulfur- or hydrogen-oxidizing, aerobic or microaerophilic bacteria.

The first group includes autotrophic bacteria of the genera *Thiomicrospira*, *Beggiatoa*, and *Thiobacillus* that fix carbon by the Calvin–Benson–Bassham cycle; these autotrophs are phylogenetic members of γ-Proteobacteria. The obligately autotrophic, aerobic, sulfur-oxidizing and mesophilic species of the genus *Thiomicrospira* are found in hydrothermal environments and sulfide-rich sediment–water interfaces worldwide. The filamentous sulfur oxidizers of the genus *Beggiatoa* form extensive mats on sediments and chimneys; most hydrothermal vent *Beggiatoa* spp. accumulate their electron acceptor nitrate intracellularly and may be facultative or obligate autotrophs.

The second group consists of mesophilic or moderately thermophilic sulfur and hydrogen oxidizers that assimilate carbon through the reverse TCA cycle; these bacteria use oxygen, nitrate, sulfur, and sulfite as the terminal electron acceptor. Historically, this group was discovered and studied in detail only after the RuBisCO (ribulose-1,5-bisphosphate carboxylase–oxygenase) autotrophs. Numerous new genera (*Caminibacter*, *Hydrogenimonas*, *Lebetimonas*, *Nautilia*, *Nitratiruptor*, *Sulfurimonas*, *Sulfurovum*) and novel species (candidatus '*Arcobacter sulfidicus*'), almost exclusively from hydrothermal vent habitats, have been described in recent years. They are members of the ε-Proteobacteria and are related to epibionts of vent invertebrates, such as the polychaete worm *Alvinella pompeiana* and the vent shrimp *Rimicaris exoculata*. The ε-Proteobacterial autotrophs generally colonize areas with higher temperatures, higher concentrations of reduced sulfur, and lower concentrations of dissolved oxygen than the γ-Proteobacterial autotrophs. This environmental preference matches the biochemical characteristics of the reverse TCA cycle; under conditions of oxygen limitation or anaerobiosis, CO_2 fixation by the reverse TCA cycle is energetically more efficient than through the Calvin cycle.

The third group consists of thermophilic and hyperthermophilic hydrogen-oxidizing bacteria of the Aquificales phylum. The hydrothermal vent Aquificales fall into the obligately hydrogen-oxidizing genera *Desulfurobacterium*, *Balnearium*, and *Thermovibrio* and the hydrogen-, sulfur-, sulfite-, and thiosulfate-oxidizing genus *Persephonella*. Oxygen, nitrate, or elemental sulfur serve as terminal electron acceptors for hydrogen oxidation. As far as is known, the Aquificales assimilate carbon through the reverse TCA cycle. The members of the Aquificales grow in temperature ranges from 45 to 95 °C, and thus overlap with the temperature range of the hyperthermophilic archaea. The Aquificales have a wide habitat range, including chimneys and sediments of deep-sea hydrothermal vents (**Figure 2**), as well as shallow-water marine vents and terrestrial hot springs.

In addition to these major physiological and phylogenetic groups of vent bacteria, other types of chemolithoautotrophic bacteria thrive at hydrothermal vents, such as the recently discovered obligate hydrogen oxidizers *Thermodesulfobacterium hydrogenophilum* and *Thermodesulfatator indicus*, both sulfate reducers and members of a phylum-level deeply branching lineage of sulfate-reducing bacteria, *Thermodesulfobacterium.* The potential for discovery of novel chemosynthetic bacteria is certainly not exhausted; molecular surveys demonstrate that only a small portion of the total bacterial diversity in hydrothermal vent sites has been cultured to date.

Symbiotic Chemolithoautotrophic Bacteria

Symbioses of sulfur-oxidizing chemolithoautotrophic bacteria with hydrothermal vent animals are characteristic of the vent ecosystem; the host animals can harbor symbionts within their bodies in specialized cells or tissues, or the symbionts live as epibionts on the exterior of the vent animals. Good examples for epibionts are the filamentous ε-Proteobacteria that grow on the carapace of the vent shrimp *R. exoculata* or on the dorsal bristles of the annelid worm *A. pompeiana*. Ongoing metagenomic analyses of the *Alvinella*-associated epibiont community reveal the potential for CO_2 fixation though the reductive TCA cycle.

Endosymbiontic sulfur-oxidizing chemolithoautotrophs have led to major modifications in the body plans of their host animals, notably in the relative size of their symbiont-bearing organs and their digestive systems. The digestive systems are greatly reduced or have completely disappeared, while other tissues have been modified to harbor the bacterial symbionts. Symbiotic bivalves provide a good example for this adaptation: Hydrothermal vent bivalves have evolved thickened gill tissues (subfilamental tissues) to accommodate their sulfur-oxidizing intracellular symbionts. As far as detailed studies are available, the sulfur-oxidizing bivalve symbionts assimilate carbon via RuBisCO. Dual symbioses are possible; bivalves of the family Mytilidae, which colonize methane-rich seeps, also harbor aerobic methane-oxidizing symbionts in addition to sulfur oxidizers. The two symbionts coexist not just within a single animal but within the same individual host cells; they can adjust their relative dominance in response to the availability of their electron donors, sulfide and methane.

The most conspicuous hydrothermal vent animal, the large tubeworm *R. pachyptila*, is a model system for the study of its unusually versatile symbionts. *R. pachyptila* houses its sulfur-oxidizing endosymbionts in a unique, richly vascularized body tissue, the trophosome, which is simultaneously supplied with oxygen, sulfide, and CO_2. These sulfur-oxidizing symbionts show an unusual mixture of metabolic strategies; they harbor RuBisCO, indicating carbon fixation via the Calvin cycle; they harbor functioning enzymes of the energy-generating TCA cycle; and they also express the enzymes of the reverse TCA cycle. Thus, the symbionts could switch from RuBisCO autotrophy to heterotrophic oxidation of carbon storage compounds via the TCA cycle to autotrophic growth via the reverse TCA cycle.

Heterotrophic Vent Bacteria

The chemosynthetic ecosystem at hydrothermal vents also provides a nutritional basis for functionally and phylogenetically diversified heterotrophic microbial communities. Some families and genera of thermophilic heterotrophic vent bacteria are found consistently at hydrothermal vents. These typical vent heterotrophs include the families Thermaceae (genera *Marinithermus*, *Oceanithermus*, *Vulcanithermus*) and Thermotogaceae (genera *Thermotoga*, *Fervidobacterium*, *Thermosipho*, *Geotoga*, and *Petrotoga*), obligate organotrophs that require complex substrates (peptides and carbohydrates) or in some cases low molecular weight organic acids. Electron acceptors for members of the Thermaceae are oxygen and nitrate; oxygen is tolerated only in low concentrations. Microaerophilic growth and wide thermophilic temperature range (total range of different species, 30–80 °C) are consistent with the steep oxygen and temperature gradients of the hydrothermal vent habitat.

The Thermotogaceae are anaerobes that grow by fermentation of carbohydrates; these thermophiles are mostly found in terrestrial hot springs, deep oil wells, geothermally heated aquifers, or shallow marine vents. So far, only *Thermosipho japonicus*, a barophile that grows with thiosulfate as electron acceptor, was isolated from a deep-sea hydrothermal vent.

Some thermophilic vent bacteria are capable of lithoautotrophic as well as heterotrophic growth. A particularly interesting example is *Caldanaerobacter subterraneus* (synonymous with *Carboxydobrachium pacificum*), a Gram-positive bacterium and member of the Firmicutes phylum that can grow autotrophically by CO oxidation in addition to anaerobic fermentative growth on complex proteinaceous substrates, such as peptone or yeast extract, or polymeric or monomeric sugars. Anaerobic fermentation of peptides and sugars is the predominant metabolism of heterotrophic, thermophilic Gram-positive bacteria of the genera *Caloranaerobacter*, *Caminicella*, *Tepidibacter*, and *Caldanerobacter*, all moderately thermophilic members of the Firmicutes phylum with a growth temperature range of 33–65 °C. Its higher growth temperature range (50–80 °C) distinguishes *Caldanaerobacter subterraneus* from other hydrothermal vent Firmicutes. Interestingly, this bacterium shares the ability for CO oxidation with a hyperthermophilic archaeon, *Thermococcus* strain AM4 that grows at temperatures of 45–95 °C (see **Table 1**). Most likely, increased heat tolerance is an adaptation to CO oxidation, since CO is a major gas constituent of hydrothermal vent end member fluids.

Hyperthermophilic Archaea

Soon after the discovery of hydrothermal vents in 1977, hyperthermophilic archaea were isolated from hydrothermal vent samples. Most archaea are anaerobes that use nitrate, oxidized metals, elemental sulfur, sulfite, sulfate, or CO_2/carbonate as electron acceptors. **Table 1** shows the diversity of currently known, metabolically and phylogenetically distinct hydrothermal vent archaea. The preferred archaeal habitats are the interior matrix of hot chimney walls, hydrothermally heated sediments, and the shallow subsurface of the porous basalt underneath hydrothermal vent areas. In these environments, conductive cooling and seawater–vent fluid mixing generate steep chemical and temperature gradients, and provide a suite of electron acceptors for anaerobic metabolism within the growth temperature range of hyperthermophilic archaea, ~80–120 °C (**Figure 3**). Under these conditions, anaerobic reducing reactions are energetically more favorable than aerobic sulfur oxidation, which dominates at mesophilic conditions up to ~50 °C. Thus, thermodynamic constraints in combination with metabolic specialization account for the dominance of archaea in hot vent habitats, for example, within chimney walls, and for the dominance of bacteria in warm or moderately hot vent environments, for example, the seawater-exposed surface of chimneys.

Chemolithoautotrophic Archaea

Autotrophic CO_2/CO assimilation is widespread among hyperthermophilic vent archaea and can be found in combination with the entire spectrum of anaerobic metabolism: hydrogen, reduced sulfur species, CO, and reduced metals serve as electron donors for respiration with nitrate, oxidized metals, elemental sulfur, sulfite and sulfate, and methanogenic reduction of CO_2 (**Table 1**). The current temperature limit for methanogenesis and

Table 1 Selected deep-sea hydrothermal vent archaea

Archaeon (genus)	*Metabolic type*	*Reaction*	*Temperature range and optimum*	
Lithoautotrophs				
Methanopyrus kandleri	Methanogenesis	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	84–110 °C	98 °C
Methanocaldococcus jannaschii	Methanogenesis	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	50–86 °C	85 °C
Archaeoglobus profundus[a]	Sulfate reduction	$4H_2 + H_2SO_4 \rightarrow H_2S + 4H_2O$	65–90 °C	82 °C
Archaeoglobus veneficus	Sulfite reduction	$3H_2 + H_2SO_3 \rightarrow H_2S + 3H_2O$	65–85 °C	75–80 °C
Pyrodictium occultum	Sulfur reduction	$H_2 + S^0 \rightarrow H_2S$	82–110 °C	105 °C
Ignicoccus pacificus	Sulfur reduction	$H_2 + S^0 \rightarrow H_2S$	70–98 °C	90 °C
Geoglobus ahangari	Iron reduction	$H_2 + 2Fe^{3+} \rightarrow 2H^+ + 2Fe^{2+}$	65–90 °C	88 °C
Geogemma strain 121	Iron reduction	$H_2 + 2Fe^{3+} \rightarrow 2H^+ + 2Fe^{2+}$	80–121 °C	105–107 °C
Pyrolobus fumarii	Nitrate and oxygen respiration	$4H_2 + NO_3^- + H^+ \rightarrow NH_3 + 3H_2O$ $2H_2 + O_2 \rightarrow 2H_2O$ (microaerophilic)	90–113 °C	106 °C
Thermococcus strain AM4	CO oxidation	$CO + H_2O \rightarrow CO_2 + H_2$	45–95 °C	82 °C
Ferroglobus placidus[b]	Fe^{2+}, S^{2-} oxidation with NO_3^-	$S^{2-} + NO_3^- + 2H^+ \rightarrow 2NO_2^- + S^0 + H_2O$ $2Fe^{2+} + NO_3^- + 2H^+ \rightarrow NO_2^- + 2Fe^{3+} + H_2O$	65–95 °C	85 °C
Heterotrophs				
Thermococcus guaymasensis	reduce S^0 during fermentation of protein-rich substrates, and produce H_2S + org. acids + CO_2; without S^0, H_2 is produced		50–90 °C	80 °C
Pyrococcus abyssi			67–102 °C	96 °C
Palaeococcus ferrophilus	reduces Fe^{3+} during fermentation of proteinaceous substrates		60–88 °C	83 °C
Aeropyrum camini	obligately aerobic heterotroph, grows on proteinaceous substrates		70–100 °C	90–95 °C
Pyrodictium abyssi	fermentation to organic acids + CO_2; growth stimulated by H_2		80–110 °C	97 °C
Staphylothermus marinus	reduces S^0 during fermentation of protein-rich substrates and produces H_2S + org. acids + CO_2		65–98 °C	92 °C

[a] Obligate H_2 oxidizer, but requires acetate as carbon source for mixotrophic growth.

[b] Isolated from a shallow-water vent.

Reproduced from Jannasch HW (1995) Microbial interactions with hydrothermal fluids. In: Humphris SE, Zierenberg RA, Mullineaux LS, and Thomson RE (eds.) *Seafloor Hydrothermal Systems: Physical, Chemical, Biological and Geological Interactions. Geophysical Monograph*, vol. 91, pp. 273–296. Washington, DC: American Geophysical Union; Miroshnichenko ML and Bonch-Osmolovskaya EA (2006) Recent developments in the thermophilic microbiology of deep-sea hydrothermal vents. *Extremophiles* 10: 85–96; Reysenbach AL, Götz D, and Yernool D (2002) Microbial diversity of marine and terrestrial thermal springs. In: Staley J and Reysenbach AL (eds.) *Biodiversity of Microbial Life*, pp. 345–421. New York: Wiley-Liss.

elemental sulfur reduction is 110 °C, the upper limit for the methanogen *Methanopyrus kandleri* and the sulfur reducer *Pyrodictium occultum*. The upper limit for sulfate reduction appears to be 90 °C, the highest growth temperature for *Archaeoglobus profundus*. Some archaeal metal reducers and nitrate reducers have even higher growth temperatures, as demonstrated by the isolation of the nitrate-reducing, microaerophilic autotrophic archaeon *Pyrolobus fumarii* with a maximal growth temperature of 113 °C and the iron (Fe-III)-reducing archaeon '*Geogemma*' with a maximal growth temperature of 121 °C. Thermodynamic stability considerations of essential macromolecules, such as proteins and DNA, in aqueous solution place the upper temperature limit of life near 130 °C; at higher temperatures, cellular macromolecules have half-life times of seconds and cannot be replenished as quickly as they are destroyed.

On the other end of the temperature spectrum, hyperthermophilic vent archaea are also found suspended in the buoyant plumes of hydrothermal fluids that are mixed into seawater and extend hundreds of meters upward into the cold, oxygenated deep-sea water column. The origin of these archaea is most likely the shallow subsurface underneath the vents and the basalt surface, where subsurface mixing of vent fluids and seawater creates a patchwork of niches with suitable environmental conditions. These subsurface reservoirs can be discharged abruptly in major volcanic eruptions; large quantities of hydrothermal fluids and gases emerge as megaplumes that can reach heights of 1–2 km and remain detectable for months. After dilution with seawater, these water masses are only minimally warmer than the deepwater background, and are fully oxygenated; yet some vent archaea survive in this environment for several weeks or months, facilitating dispersal.

Heterotrophic Archaea

The majority of currently described archaeal hyperthermophilic isolates from hydrothermal vents are heterotrophic. The mutually related genera *Thermococcus* and *Pyrococcus* are the most frequently isolated representatives; they assimilate and ferment complex organic compounds such as yeast extract, tryptone, peptone, casein, diverse sugars, and peptides. Fermentative growth is enhanced by the addition of elemental sulfur, or requires sulfur as an essential component in some species; sulfur acts as an auxiliary electron acceptor, and hydrogen sulfide accumulates in high concentrations. Members of the genera *Thermococcus* and *Pyrococcus* grow quickly and robustly on a wide range of liquid, anaerobic laboratory media supplemented with sulfur, and are isolated so frequently from all kinds of hydrothermal vent sample materials, including the subsurface underneath hydrothermal vents, that they are viewed as indicator species of hydrothermal activity.

Thermococcus and *Pyrococcus* spp. are particularly interesting as model archaea that can grow as biofilms, often with specific adaptation to chemical or physiological stress. In laboratory simulations and within the porous rock of hydrothermal chimneys, hyperthermophiles grow attached to each other and to their mineral substrate, surrounded by capsular exopolysaccharides. Mutually related cultured *Thermococcus* and *Pyrococcus* species differ slightly in temperature sensitivity, substrate spectra, and proteolytic repertoire. These physiological differences among *Thermococcus* and *Pyrococcus* isolates, and the numerous variants of their molecular marker genes (16S rRNA genes) in hydrothermal vent samples, indicate that this diversity is a consistently recurring key feature of archaeal vent communities and therefore must have an ecological explanation. Resource partitioning within the chimney matrix is the most likely explanation, analogous to other microbial ecosystems in hot springs and nonthermal habitats.

Uncultured Archaea and Bacteria at Hydrothermal Vents

Molecular surveys based on cloning and sequencing of 16S rRNA and functional genes have revealed an unexpected diversity of uncultured archaeal and bacterial lineages at hydrothermal vents. These lineages lack cultured relatives that could serve as a baseline for physiological inferences or cultivation strategies. Obviously, the evolutionary and physiological diversity of hydrothermal vent microorganisms has been explored only to a limited extent; metagenomic studies and increased cultivation efforts would enable significant progress. Many deeply branching archaeal and bacterial lineages at hydrothermal vents cannot be subsumed into the well-defined phylum-level lineages, but represent deeply branching lineages that emerge from the earliest evolutionary radiation of the bacterial and the archaeal domains. These findings have lend new support to the hypothesis that deep-sea hydrothermal vent microbial ecosystems are among the oldest on Earth, and have sustained microbial life already during its early diversification more than 3.5 billion years ago.

Comparison to Terrestrial Hot Springs

Interestingly, hydrothermal vents and terrestrial hot springs harbor very distinct microbial communities, as a consequence of key differences in the chemical regime. Specific archaeal and bacterial groups show strong biases in environmental preference and can be viewed as signature communities for deep-sea vents and terrestrial hot springs.

The archaeal composition of terrestrial hot springs and deep-sea vents reflects differences in sulfur cycle chemistry and pH. Terrestrial hot springs with reduced sulfur species turn acidic due to proton-releasing sulfur oxidation reactions, once the limited local buffering capacity is exhausted. In contrast, the acidic pH of end member fluids in marine hydrothermal vents is quickly attenuated by mixing with slightly basic seawater, which also buffers protons released by microbial sulfur oxidation reactions. Therefore, terrestrial hot springs harbor a great diversity of acidophilic sulfur-oxidizing archaea (genera *Sulfolobus*, *Acidianus*, *Sulfurococcus*, *Metallosphaera*) that have so far not been found in deep-sea hydrothermal vents; conversely, the neutrophilic sulfur reducers of the genera *Thermococcus* and *Pyrococcus* provide examples for almost exclusively marine archaea that are largely missing in terrestrial hot springs. Around 20 recognized *Thermococcus* spp. and a large number of strains have been isolated from marine, deep and shallow hydrothermal vents; only two species have been described from a volcanic lake in New Zealand.

The second example for strong habitat preference, in favor of terrestrial hot springs and against deep-sea hydrothermal vents, is provided by the Thermotogales, a deeply branching lineage of thermophilic, heterotrophic bacteria. Only *T. japonicus*, a barophile that grows with thiosulfate as electron acceptor, was isolated from a deep-sea hydrothermal vent. Some members of the Thermotogales are found exclusively in the terrestrial subsurface (genus *Geotoga*) or in freshwater hot springs (genus *Fervidobacterium*). The ecophysiologically diversified genus *Thermotoga* includes species isolated from shallow-water marine vents, hot springs in salt lakes, and the geothermally heated terrestrial subsurface. In many cases, the freshwater representatives of *Thermotoga* are more sensitive to high salt concentrations and to high sulfur concentrations. The Thermotogales' preference for sugars and carbohydrates as carbon and energy sources might also link them to terrestrial habitats and hot springs where plant-derived carbon substrates are available.

The Aquificales are one of the very few groups of hyperthermophiles that occur as a dominant group in deep-sea hydrothermal vents and shallow-water vents, as well as in terrestrial hot springs; they are at home in both worlds. For example, the Aquificales are the dominant hyperthermophilic autotrophic bacteria in the near-neutral Yellowstone hot springs. Here, hydrogen instead of sulfide is the dominant inorganic electron donor for chemolithoautotrophic metabolism; atmospheric oxygen is readily available for microaerophilic growth with hydrogen.

At temperatures below 72–74 °C, photoautotrophic bacteria, such as thermophilic cyanobacteria, gain a foothold and turn hot springs into photoautotrophic microbial ecosystems where the community structure of thermophilic cyanobacteria, and the ecophysiological specialization of specific cyanobacterial community members, reflects the physical and chemical zonation of the microbial habitat.

Unusual Hydrothermal Vents: Loihi, Guaymas, Lost City

Unusual geological and chemical settings result in several unique hydrothermal vent systems that are very different from the classical mid-ocean ridge, black smoker-type hydrothermal vents. The unique geochemistry of these vents is reflected in their unusual microbial communities. The surprising diversity of geologically, geochemically, and microbiologically different vent sites is illustrated here with three unusual vent sites: The Loihi vents southeast of Hawaii, a vent field dominated by iron-oxidizing microbial communities on a growing seamount that will emerge as the next Hawaiian Island; the Guaymas Basin vents in the Gulf of California, where a mid-ocean spreading center is buried under thick organic-rich sediments whose organics undergo fast thermal maturation; and the Lost City vents located off-axis near the Mid-Atlantic Ridge, where nonvolcanic rock–water chemistry provide the inorganic electron donors for microbial life in huge carbonate chimneys.

The Loihi vents are located at the top of Loihi Seamount on the southeastern end of the Hawaiian Island chain at 900 m depth; the vent fluids are dominated by high concentrations of dissolved reduced iron and CO_2, but contain little or no sulfide; their temperature range is less extreme (10–170 °C) than that at most mid-ocean ridge sites. The iron-rich vent fluids of Loihi emerge into the oxygen minimum layer of the marine water column, which slows down abiogenic iron oxidation in oxygenated seawater and favors the development of extensive iron-oxidizing bacterial communities. The iron-oxidizing microbial communities of Loihi are dominated by mesophilic or psychrophilic, microaerophilic, and neutrophilic Fe-II-oxidizing bacteria that grow in thick, rust-colored microbial mats of freshly produced and precipitated amorphous iron oxides; the bacteria are members of the different Proteobacterial subdivisions (**Figure 4**). Phylogenetically and physiologically similar Fe-II-oxidizing bacteria have also been isolated from

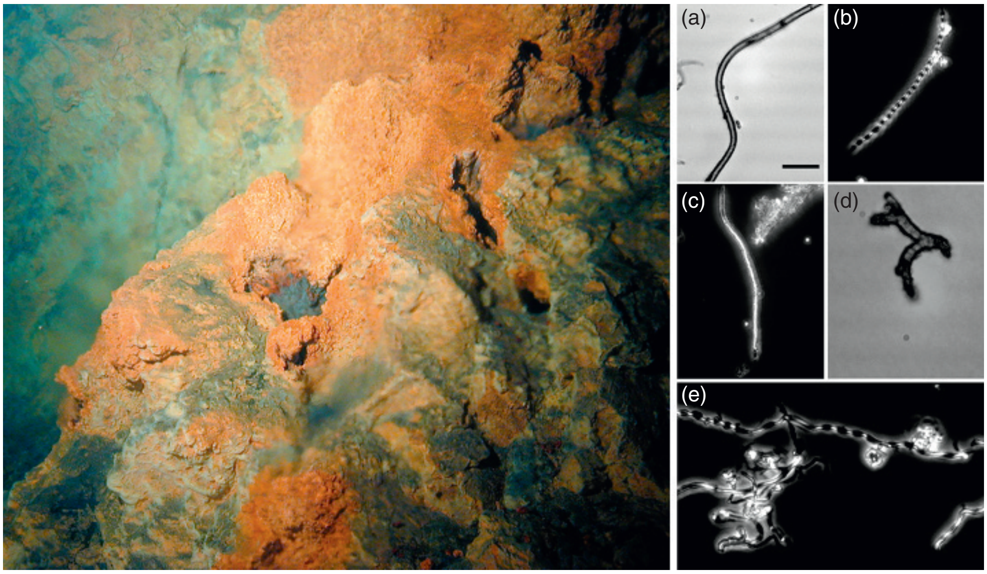

Figure 4 Hydrothermal vent orifices at Loihi Seamount surrounded by rust-colored microbial mats that are responsible for depositing iron oxides (left panel). These vents are located at a depth of approximately 1300 m in a caldera near the summit of Loihi. The anoxic vent fluid contains 100 μm Fe(II) and is rich in CO_2, but has a very low sulfide content; vent fluid temperature at the time of this photograph was approximately 60 °C. The vents support a robust community of iron-oxidizing bacteria. The right hand panel shows light photomicrographs documenting the morphology of biogenically produced iron oxides typically found in these mat communities. (a) Tubular sheath encrusted with iron oxides; filaments of cells are sometimes visible in these when samples are stained with a fluorescent DNA stain. (b), (c), and (e) show twisted, filamentous stalk-like structures that are formed by a novel member of the Proteobacteria Reproduced from Emerson D and Moyer CL (2002) Neutrophilic iron-oxidizing bacteria are abundant at the Loihi Seamount hydrothermal vents and play a major role in Fe oxide deposition. *Applied and Environmental Microbiology* 68: 3085–3093. (d) Y-shaped morphology of dense iron-oxides formed by cells that grow at the apical tips of the oxide structures. The scale bar corresponds to 5 μm. Photo credits: Vent: Kerby T, Hawaiian Undersea Research Laboratory; photomicrographs: Emerson D.

other vent sites during *in situ* colonization experiments with metal sulfides.

The Guaymas Basin vents in the Gulf of California, between Baja California and the Mexican Mainland, are a mid-ocean ridge that is buried under hundreds of meters of organic-rich sediments that originate from high primary productivity in the upper water column, and from terrestrial runoff. As the vent fluids percolate upward through the sediment layers, the buried organic material in the sediments is geothermally heated and matures to petroleum compounds, including complex aromatics, organic acids, alkanes, and methane. Nitrogen compounds are reduced to ammonia, absent in most vent fluids but abundant at Guaymas. Most of the dissolved metals are precipitated *en route* to the sediment surface, which reduces the metal toxicity of the fluids. The usually acidic pH of the vent fluids is buffered to the near-neutral range by buried carbonates. The resulting organic cocktail sustains extensive communities of anaerobic archaea and bacteria in the sediment, including methanogens, sulfate-dependent methane oxidizers, sulfate reducers, and anaerobic heterotrophs. Sulfide and dissolved inorganic carbon in the vent fluids sustain massive sulfur-oxidizing microbial mats of filamentous *Beggiatoa* spp. at the sediment surface (**Figure 5**). The methane in the vent fluids sustains anaerobic methane-oxidizing (ANME) communities in the surficial sediments. Here, methane is oxidized by novel archaea in a consortium with sulfate-reducing bacteria that transfer the methane-derived electrons on sulfate as the electron acceptor. Overall, the Guaymas sediment communities are more similar to those of petroleum and methane seeps than to standard mid-ocean ridge vent sites.

The Lost City hydrothermal vent field on the flanks of the Mid-Atlantic Ridge owes its existence to deep subsurface mineral–fluid reactions unlike those of mid-ocean spreading centers. The dominant type of rock–fluid interactions are serpentinization reactions in the earth's crust that produce hydrogen and methane; the resulting vent fluids are warm or moderately hot (40–80 °C) and contain 2 orders magnitude less sulfide than hot vent fluids from mid-ocean ridges. The high pH of 9–10 and the high Ca^{2+} content of the vent fluids lead to precipitation of large carbonate mounds, pinnacles, and chimneys, unlike the previously known metal sulfide chimneys; with a height of 40–60 m, the Lost City structures are the largest known hydrothermal chimneys.

The microbial community of the Lost City vents indicates an active microbial methane and sulfur cycle. The archaeal communities of the Lost City include members of the Methanosarcinales, uncultured methanogens that form biofilms on surfaces exposed to the hottest vent fluids at the Lost City (~80 °C) where they have access to molecular hydrogen. Cooler areas are dominated by anaerobic methane-oxidizing archaea (ANME-1 group) that gain energy by sulfate-dependent oxidation of

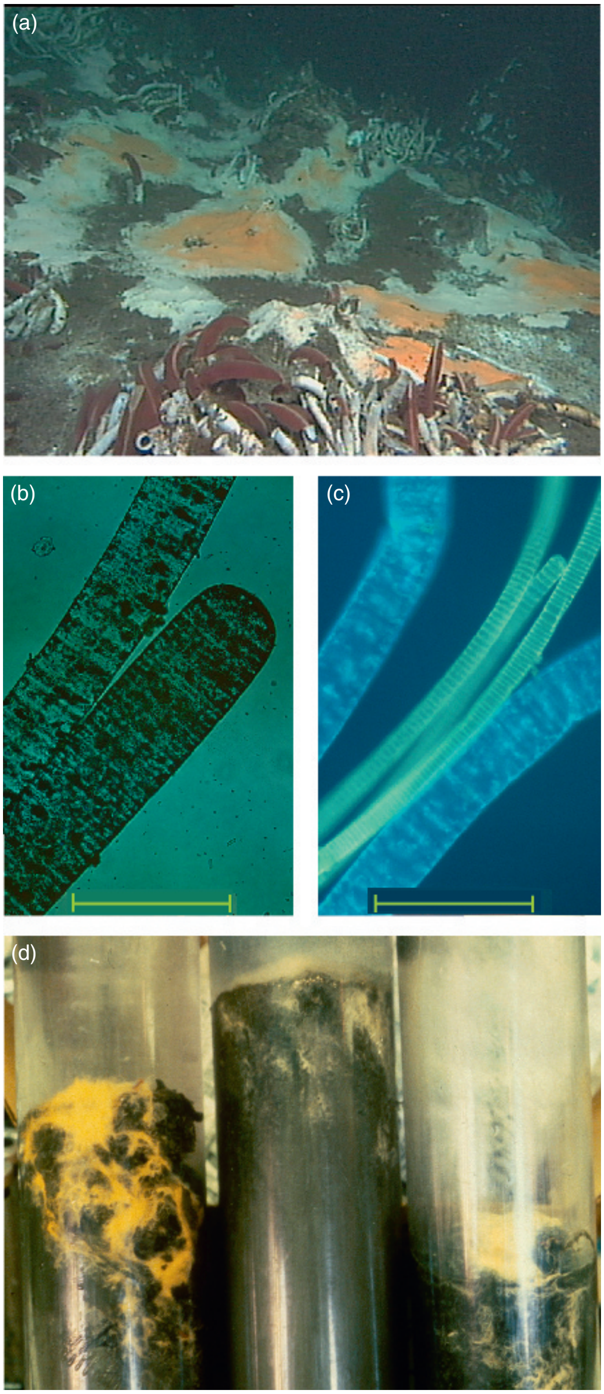

Figure 5 Guaymas Basin. (a) White and orange *Beggiatoa* mats and a colony of *Riftia pachyptila*, at Guaymas Basin. The diameter of the mats is ~1 feet. Photo credits: Shank T, WHOI. (b) Transmission light microphotograph of the large white *Beggiatoa* spp., showing the cells within the filaments. Scale bars, 250 um. Photo credits: Teske A. (c) Epifluorescence light microphotograph of white and orange *Beggiatoa* spp., under UV excitation light. The larger white *Beggiatoa* spp. appears bluish and the smaller orange *Beggiatoa* spp. appears yellow-orange. Photo credits: Teske A. (d). Freshly retrieved push cores (2.5 inch diameter) with hydrothermal sediment, covered with mats of orange *Beggiatoa* spp. Photo credits: Jannasch HW and Nelson D.

methane. The bacterial communities predominantly include the γ- and ε-Proteobacteria and Firmicutes, in addition to a diverse assemblage of Chloroflexi, Planctomyces, Actinobacteria, Nitrospira, and uncultured subsurface lineages. The γ-Proteobacteria at the Lost City vents are closely related to the cultured members of the genera that aerobically oxidize sulfide (*Thiomicrospira*) and methane (*Methylomonas* and *Methylobacter*). Uncultured ε-Proteobacteria are found as well and are most likely involved in hydrogen and sulfide oxidation. Several uncultured members of the Firmicutes are most closely related to members of the sulfate-reducing genus *Desulfotomaculum.* Thus, the molecular surveys indicate a fully formed anaerobic archaeal methane cycle of hydrogenotrophic methanogens and sulfate-dependent methane oxidizers at the Lost City vents. Bacterial communities oxidize methane aerobically, and also constitute a full sulfur cycle with anaerobic Gram-positive sulfate reducers and aerobic or microaerophilic sulfide oxidizers; hydrogen, methane, and sulfide sustain the chemosynthetic microbial communities at the Lost City vents.

The Lost City vent site is the first example of a previously unknown, chemically distinct and unusually long-lived type of hydrothermal vent; the serpentinization reactions in the vent subsurface can yield energy and microbial electron donors over hundreds of thousands of years. Radiocarbon tests of the vent carbonates have shown that the Lost City vents have been active for at least 30 000 years. This long-term habitat stability, and the wide distribution of geological settings that are favorable for serpentinization reactions, has major implications for the evolution of microbial life on Earth and on other planets.

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DNA Restriction and Modification

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Glossary

ATP and ATP hydrolysis Adenosine triphosphate (ATP) is a primary repository of energy that is released for other catalytic activities when ATP is hydrolyzed (split) to yield adenosine diphosphate (ADP).

bacteriophage **(λ and T-even)** Bacterial viruses. Phage lambda (λ) is a temperate phage, and therefore on infection of a bacterial cell, one of two alternative pathways may result; either the lytic pathway in which the bacterium is sacrificed and progeny phage are produced, or the temperate (lysogenic) pathway in which the phage genome is repressed and, if it integrates into the host chromosome, will be stably maintained in the progeny of the surviving bacterium. Phage λ was isolated from *Escherichia coli* K-12 in which it resided in its temperate (prophage) state. T-even phage (T2, T4, and T6) are virulent coliphage, that is, infection of a sensitive strain of *E. coli* leads to the production of phage at the inevitable expense of the host. T-even phage share the unusual characteristic that their DNA includes hydroxymethylcytosine rather than cytosine.

DNA methyltransferases These enzymes (MTases) catalyze the transfer of a methyl group from the donor *S*-adenosylmethionine (AdoMet or SAM) to adenine or cytosine residues in the DNA.

efficiency of plating (EOP) This usually refers to the ratio of the plaque count on a test strain relative to that obtained on a standard, or reference, strain.

endonucleases Enzymes that can fragment polynucleotides by the hydrolysis of internal phosphodiester bonds.

Escherichia coli *strain K-12* The strain used by Lederberg and Tatum in their discovery of recombination in *E. coli*.

glucosylation of DNA The DNA of T-even phage in addition to the pentose sugar, deoxyribose, contains glucose attached to the hydroxymethyl group of hydroxymethylcytosine. Glucosylation of the DNA is mediated by phage-encoded enzymes, but the host provides the glucose donor.

helicases Enzymes that separate paired strands of polynucleotides.

recombination pathway The process by which new combinations of DNA sequences are generated. The general recombination process relies on enzymes that use DNA sequence homology for the recognition of the recombining partner. In the major pathway in *E. coli* the RecBCD enzyme, also recognized as exonuclease V, enters DNA via a double-strand break. It tracks along the DNA, promoting unwinding of the strands and DNA degradation. The activity of RecBCD changes when it encounters Chi, a special DNA sequence in the 3′ to 5′ orientation. Degradation at the 5′ end is now favored and the single-stranded DNA with a 3′ end becomes a substrate for RecA-mediated strand transfer into an homologous DNA duplex.

SOS response DNA damage induces expression of a set of genes, the SOS genes, involved in the repair of DNA damage.

Southern transfer The transfer of denatured DNA from a gel to a solid matrix, such as a nitrocellulose filter, within which the denatured DNA can be maintained and hybridized to labeled probes (single-stranded DNA or RNA molecules). Fragments previously separated by electrophoresis through a gel may be identified by hybridization to a specific probe.

transformation The direct assimilation of DNA by a cell, as the result of which the recipient is changed genetically.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
EOP	efficiency of plating
HMC	hydroxymethylcytosine
PCR	polymerase chain reaction
R-M	restriction and modification
SAM	S-adenosylmethionine
TRDs	target recognition domains

Introduction

Awareness of the biological phenomenon of restriction and modification (R-M) grew from the observations of microbiologists that the host range of a bacterial virus (phage) was influenced by the bacterial strain in which the phage was last propagated. Although phage produced in one strain of *Escherichia coli* would readily infect a culture of the same strain, they might only rarely achieve the successful infection of cells from a different strain of *E. coli.* This finding implied that the phage carried an 'imprint' that identified their immediate provenance. Simple biological tests showed that the occasional successful infection of a different strain resulted in the production of phage that had lost their previous imprint and had acquired a new one, that is, they acquired a different host range.

In the 1960s, elegant molecular experiments showed the 'imprint' to be a DNA modification that was lost when the phage DNA replicated within a different bacterial strain; those phage that conserved one of their original DNA strands retained the imprint, or modification, whereas phage containing two strands of newly synthesized DNA did not. The modification was shown to provide protection against an endonuclease, the barrier that prevented the replication of incoming phage genomes. The host-controlled barrier to successful infection by phage that lacked the correct modification was referred to as 'restriction' and the relevant endonucleases have acquired the colloquial name of restriction enzymes. The modification enzyme was shown to be a DNA methyltransferase that methylated specific bases within the target sequence, and in the absence of the specific methylation, the target sequence rendered the DNA sensitive to the restriction enzyme. When DNA lacking the appropriate modification imprint enters a restriction-proficient cell, it is recognized as foreign and cut by the endonuclease. Classically, a restriction enzyme is accompanied by its cognate modification enzyme and the two comprise an R-M system. Most restriction systems conform to this classical pattern. There are, however, some restriction endonucleases that attack DNA only when their target sequence is modified. A restriction system that responds to its target sequence only when it is identified by modified bases does not, therefore, coexist with a cognate modification enzyme.

Two early papers documented the phenomenon of restriction. In one, Bertani and Weigle, in 1953, using temperate phage (λ and P2), identified the classical R-M systems characteristic of *E. coli* K-12 and *E. coli* B. In the other, Luria and Human, in 1952, identified a restriction system of a nonclassical kind. In the experiments of Luria and Human, T-even phage were used as test phage and after their growth in a mutant *E. coli* host they were found to be restricted by wild-type *E. coli* K-12, but not by *Shigella dysenteriae.* An understanding of the restriction phenomenon observed by Luria and Human requires knowledge of the special nature of the DNA of T-even phage. During replication of T-even phage, the unusual base 5-hydroxymethylcytosine (HMC) completely substitutes for cytosine in the T-phage DNA, and hydroxymethyl residues become substrates for glucosylation. In the mutant strain of *E. coli* used by Luria and Human as host for the T-even phage, glucosylation fails and, in its absence, the nonglucosylated phage DNA becomes sensitive to endonucleases present in *E. coli* K-12 but not in *S. dysenteriae*; particular nucleotide sequences normally protected by glucosylation are recognized in *E. coli* when they include the modified base, HMC, rather than cytosine residues. These endonucleases, now accepted as restriction systems, were later discovered to attack DNA that includes methylated cytosine residues. Strains lacking these endonucleases enhanced the efficiency of cloning foreign DNA in *E. coli.*

The classical R-M systems and the modification-dependent restriction enzymes share the potential to attack DNA derived from different strains and thereby 'restrict' DNA transfer. They differ in that in one case an associated modification enzyme is required to protect DNA from attack by the cognate restriction enzyme and in the other modification enzymes specified by different strains impart signals that provoke the destructive activity of restriction endonucleases.

Detection of Restriction Systems

As a Barrier to Gene Transfer

This is exemplified by the original detection of the R-M systems of *E. coli* K-12 and *E. coli* B by Bertani and Weigle in 1953. Phage λ grown on *E. coli* strain C (λ.C), where

E. coli C is a strain that apparently lacks an R-M system, forms plaques with poor efficiency (EOP of 2×10^{-4}) on *E. coli* K-12 because the phage DNA is attacked by a restriction endonuclease (**Figure 1**). Phage λ grown on *E. coli* K-12 (λ.K) forms plaques with equal efficiency on *E. coli* K-12 and *E. coli* C, since it has the modification required to protect against the restriction system of *E. coli* K-12 and *E. coli* C has no restriction system (**Figure 1**). In contrast, λ.K will form plaques with very low efficiency on a third strain, *E. coli* B, since *E. coli* B has an R-M system with different sequence specificity from that of *E. coli* K-12.

Phage often provide a useful and sensitive test for the presence of R-M systems in laboratory strains of bacteria, but they are not a suitable vehicle for the general detection of barriers to gene transfer. Many bacterial strains, even within the same species and particularly when isolated from natural habitats, are unable to support the propagation of the available test phage and some phage (e.g., P1) have the means to antagonize at least some restriction systems (see 'Antirestriction systems'). Gene transfer by conjugation can monitor restriction, although some natural plasmids, but probably not the F factor of *E. coli*, are equipped with antirestriction systems. The single-stranded DNA that enters a recipient cell by conjugation, or following infection by a phage such as M13, becomes sensitive to restriction only after the synthesis of its complementary second strand. In contrast, the single-stranded DNA that transforms naturally competent bacteria may not become a target for restriction because it forms heteroduplex DNA with resident (and therefore modified) DNA, and one modified strand is sufficient to endow protection. Transformation can be used to detect restriction systems when the target DNA is the double-stranded DNA of a plasmid.

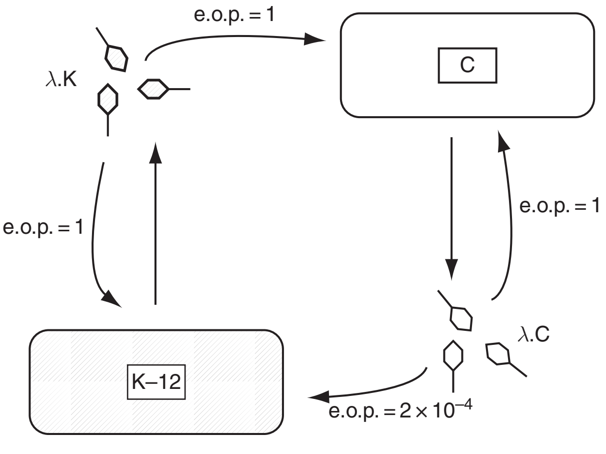

Figure 1 Host-controlled restriction of bacteriophage λ. *Escherichia coli* K-12 possesses, whereas *E. coli* C lacks, a Type I R-M system. Phage λ propagated in *E. coli* C (λ.C) is not protected from restriction by EcoKI and thus forms plaques with reduced efficiency of plating (EOP) on *E. coli* K-12 as compared to *E. coli* C. Phage escaping restriction are modified by the EcoKI methyltransferase (λ.K), and consequently form plaques with the same efficiency on *E. coli* K-12 and C. Modified DNA is indicated by hatch marks. Reproduced from Barcus VA and Murray NE (1995) Barriers to recombination: Restriction. In: Baumberg S, Young JPW, Saunders SR, and Wellington EMH (eds.) *Population Genetics of Bacteria Society for General Microbiology*, Symposium No. 52, pp. 31–58. Cambridge, UK: Cambridge University Press.

In Vitro Assays for DNA Fragmentation

Endonuclease activities yielding discrete fragments of DNA are commonly detected in crude extracts of bacterial cells. More than one substrate may be used to increase the chance of providing DNA that includes appropriate target sequences. DNA fragments diagnostic of endonuclease activity are separated according to their size by electrophoresis through a matrix, usually an agarose gel, and are visualized by the use of autoradiography or a fluorescent dye, ethidium bromide, that intercalates between stacked base pairs.

Extensive screening of many bacteria, often obscure species for which there is no genetic test, has produced a wealth of endonucleases with different target sequence specificities. These endonucleases are referred to as restriction enzymes, even in the absence of biological experiments to indicate their role as a barrier to the transfer of DNA. Many of these enzymes are among the commercially available endonucleases that serve molecular biologists in the analysis of DNA (**Table 1**; see 'Applications and commercial relevance'). *In vitro* screens are applicable to all organisms, but to date R-M systems have not been found in eukaryotes, although some algal viruses encode them.

Sequence-Specific Screens

The identification of new R-M genes via sequence similarities is sometimes possible. Only occasionally are gene sequences sufficiently conserved that the presence of related systems can be detected by probing Southern transfers of bacterial DNA. More generally, screening databases of predicted polypeptide sequences for relevant motifs has identified putative R-M systems in the rapidly growing list of bacteria for which the genomic sequence is available (see 'Distribution'). Currently, this approach is more dependable for modification methyltransferases than restriction endonucleases, but the genes encoding the modification and restriction enzymes are usually adjacent. Many putative R-M systems have been identified in bacterial genomic sequences.

Nomenclature and Classification

Nomenclature

R-M systems are designated by a three-letter acronym derived from the name of the organism in which they

Table 1 Some Type II restriction endonucleases and their cleavage sites[a]

Bacterial source	*Enzyme abbreviation*	*Sequences 5′ → 3′ 3′ ← 5′*	*Note*[b]
Haemophilus influenzae Rd	HindII	GTPy↓PuAC CAPu↑PyTG	1, 5
	HindIII	↓ AAGCTT TTCGAA ↑	2
Haemophilus aegyptius	HaeIII	GG↓CC CC↑GG	1
Staphylococcus aureus 3A	Sau3AI	↓GATC CTAG↑	2, 3
Bacillus amyloliquefaciens H	BamHI	↓ GGATCC CCTAGG ↑	2, 3
Escherichia coli RY13	EcoRI	↓ GAATTC CTTAAG ↑	2
Providencia stuartii	PstI	↓ CTGCAG GACGTC ↑	4

[a] The cleavage site for each enzyme is shown by the arrows.

[b] 1, produces blunt ends; 2, produces cohesive ends with 5′ single-stranded overhangs; 3, cohesive ends of Sau3AI and BamHI are identical; 4, produces cohesive ends with 3′ single-stranded overhangs; 5, Pu is any purine (A or G), and Py is any pyrimidine (C or T).

occur. The first letter comes from the genus, and the second and third letters from the species. The strain designation, if any, follows the acronym. Different systems in the same organism are distinguished by Roman numerals. Thus HindII and HindIII are two enzymes from *Haemophilus influenzae* strain Rd. Restriction endonuclease and modification methyltransferases (ENases and MTases) are sometimes distinguished by the prefixes R.EcoRI and M.EcoRI, but the prefix is commonly omitted if the context is unambiguous. The current convention for ENase naming omits italicization.

Classification of R-M Systems

R-M systems are classified according to the composition and cofactor requirements of the enzymes, the nature of the target sequence, and the position of the site of DNA cleavage with respect to the target sequence. Currently R-M systems are divided into three types (I, II, and III). In addition there are modification-dependent restriction systems, now referred to as Type IV. Early experiments identified Type I systems, but the Type II systems are the simplest and for this reason will be described first. A summary of the properties of different types of R-M systems is given in **Figure 2**.

Type II R-M Systems

A classical Type II R-M system comprises two separate enzymes; one is the restriction ENase, the other the modification MTase. The nuclease activity requires Mg^{2+}, and DNA methylation requires S-adenosylmethionine (AdoMet or SAM) as methyl donor. The target sequence of both enzymes is the same; the modification enzyme ensures that a specific base within the target sequence, one on each strand of the duplex, is methylated and the restriction endonuclease cleaves unmodified substrates within, or close to, the target sequence. The target sequences are often rotationally symmetrical sequences of 4–8 bp; for example, a duplex of the sequence 5′GAA*TTC is recognized by EcoRI. The modification enzyme methylates the adenine residue identified by the asterisk, but in the absence of methylated adenine residues on both strands of the target sequence the restriction endonuclease breaks the phosphodiester backbones of the DNA duplex to generate ends with 3′ hydroxyl and 5′ phosphate groups. Type II ENases cut within or close to their target sequences. The nature of the modification introduced by the MTase varies according to the system: N6-methyladenine (m6A) and N5- and N4-methylcytosine (m5C and m4C). Irrespective of the target sequence, or the nature of the modification, ENases differ in that some cut the DNA to generate ends with 5′ overhangs,

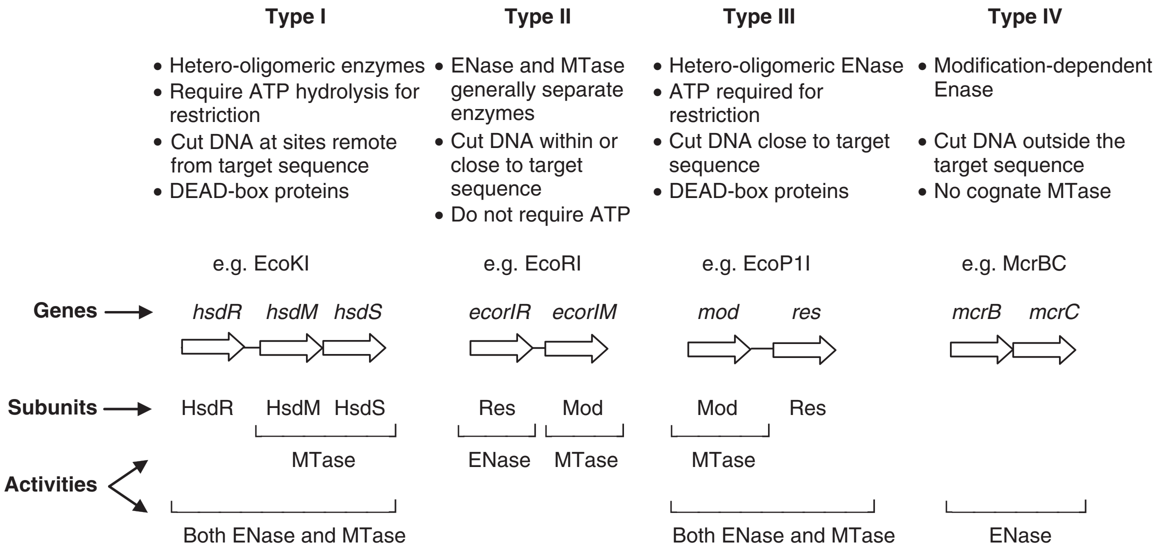
Type I
Type II
Type III
Type IV
• Hetero-oligomeric enzymes
• Require ATP hydrolysis for restriction
• Cut DNA at sites remote from target sequence
• DEAD-box proteins
• ENase and MTase generally separate enzymes
• Cut DNA within or close to target sequence
• Do not require ATP
• Hetero-oligomeric ENase
• ATP required for restriction
• Cut DNA close to target sequence
• DEAD-box proteins
• Modification-dependent Enase
• Cut DNA outside the target sequence
• No cognate MTase
e.g. EcoKI
e.g. EcoRI
e.g. EcoP1I
e.g. McrBC
Genes
hsdR hsdM hsdS
ecorIR ecorIM
mod res
mcrB mcrC
Subunits
HsdR HsdM HsdS
Res Mod
Mod Res
MTase
ENase MTase
MTase
Activities
Both ENase and MTase
Both ENase and MTase
ENase

Figure 2 The characteristics and organization of the genetic determinants and subunits of the different types of restriction systems. These systems are classified on the basis of their complexity, cofactor requirements and position of DNA cleavage with respect to their DNA target sequence. The Type I, II and III systems are the classical restriction and modification (R-M) systems. The restriction enzymes of the Type I and III systems contain motifs characteristic of 'DEAD'-box proteins. These motifs are associated with ATP-dependent DNA translocation. The Type II restriction enzymes do not translocate DNA, and their properties are sufficiently variable for them to be allocated to subclasses. Some indication of this is given in the text. Modification-dependent endonucleases are now included as Type IV restriction systems. ENase, restriction endonuclease; MTase, methyltransferase (Adapted from King and Murray, 1995).

some generate 3′ overhangs and others produce ends which are 'blunt' or 'flush' (see **Table 1**).

Classical Type II restriction enzymes are generally active as symmetrically arranged homodimers, an association that facilitates the coordinated cleavage of both strands of the DNA. In contrast, Type II modification enzymes act as monomers, an organization consistent with their normal role in the methylation of newly replicated DNA in which one strand is already methylated.

The genes encoding Type II R-M systems derive from the name of the system. The genes specifying R.BamHI and M.BamHI, for example, are designated *bamHIR* and *bamHIM*. Transfer of the gene encoding a restriction enzyme, in the absence of the transfer of the partner encoding the protective MTase, is likely to be lethal if the recipient cell does not provide the relevant protection. Experimental evidence supports the expectation that the genes encoding the two components of R-M systems are usually closely linked so that cotransfer will be efficient.

The extensive characterization of restriction endonucleases during 40 years has led to significant broadening of the Type II class, and its subsequent division into many subclasses. Subclass IIP includes classical representatives such as EcoRI and HindIII, in which the endonucleases comprise symmetrically arranged homodimers that permit recognition of a symmetrical (palindromic) target sequence, and the cutting of each strand of DNA at fixed symmetrical locations, either within the target sequence or immediately adjacent to it. DpnI, a similar dimeric endonuclease that cuts within a symmetrical target sequence, but only if methylated, is now accepted as a Type II system. It identifies subclass M (methylation-dependent). Systems that recognize asymmetric target sequences are assigned to subclass IIA, but many of these, for example, FokI, cut the DNA at a precise, but short, distance from their recognition sequence, and therefore, they also meet the requirement for subclass IIS (where S refers to the shifted position of the cut).

The endonucleases of members of the subclasses IIB, C, G, and H have hybrid structures that include both endonuclease and modification domains within a single polypeptide. The activity of members of subclasses IIE and IIF is mechanistically dependent on two target sequences. In subclass IIE one of the two targets only serves as an accessory site, while in subclass IIF both targets are substrates for coordinate cleavage.

This brief survey of even the Type II subclasses illustrates enormous variation among sequence-specific endonucleases.

Type I R-M Systems

Type I R-M systems are multifunctional enzymes comprising three subunits that catalyze both restriction and modification. In addition to Mg^{2+}, endonucleolytic activity requires both AdoMet and adenosine triphosphate (ATP). The restriction activity of Type I enzymes is associated with the hydrolysis of ATP, an activity that correlates with the peculiar characteristic of these enzymes, that of cutting DNA at nonspecific nucleotide sequences considerable distances from their target sequences. The Type I R-M enzyme binds to its target sequence and its activity as an ENase or a MTase is determined by the methylation state of the target sequence. If the target sequence is unmodified, the enzyme, while bound to its target site, is believed to translocate DNA toward itself simultaneously in both directions in an ATP-dependent manner. This translocation process brings the bound enzymes closer to each other and experimental evidence suggests that DNA cleavage occurs when translocation is impeded, either by collision with another translocating complex or by the topology of the DNA substrate.

The nucleotide sequences recognized by Type I enzymes are asymmetric and comprise two components, one of 3 or 4 bp and the other of 4 or 5 bp, separated by a nonspecific spacer of 6–8 bp. All known Type I enzymes methylate adenine residues, one on each strand of the target sequence.

The three subunits of a Type I R-M enzyme are commonly encoded by three contiguous genes: *hsdR*, *hsdM*, and *hsdS*. The acronym *hsd* was chosen at a time when R-M systems were referred to as host specificity systems and *hsd* denotes host specificity of DNA. *hsdM* and *hsdS* are transcribed from the same promoter, but *hsdR* from a separate one. The two subunits encoded by *hsdM* and *hsdS*, sometimes referred to as M and S, are both necessary and sufficient for MTase activity. The third subunit (R) is essential only for restriction. The S subunit includes two target recognition domains (TRDs) that impart target sequence specificity to both the restriction and modification activities of the complex; the M subunits include the binding site for AdoMet and the active site for DNA methylation. Two complexes of Hsd subunits are functional in bacterial cells, one that comprises all three subunits ($R_2M_2S_1$) and is an R-M system, and a second that lacks R (M_2S_1) and has only MTase activity.

Type III R-M Systems

Type III R-M systems are less complex than Type I, but nevertheless share some similarities with them. A single hetero-oligomeric complex catalyzes both the restriction and modification activities. Modification requires the cofactor AdoMet and is stimulated by Mg^{2+} and ATP. Restriction requires Mg^{2+} and ATP, and is stimulated by AdoMet. The recognition sequences of Type III modification enzymes are asymmetric sequences of 5–6 bp. Restriction requires two unmodified sequences in inverse orientation (**Figure 3(a)**). Recent evidence shows that Type III R-M enzymes, like Type I, can translocate DNA in a process dependent on ATP hydrolysis, but they hydrolyze less ATP than Type I systems, and probably only translocate DNA for a relatively short distance. Cleavage is stimulated by collision of the translocating complexes and occurs on the 3′ side of the recognition sequence at a distance of approximately 25–27 bp: this contrasts with cleavage by Type I enzymes where cutting occurs at sites remote from the recognition sequence. Because only one strand of the recognition sequence of a Type III R-M system is a substrate for methylation, it might be anticipated that the immediate product of replication would be sensitive to restriction. In order to understand why this is not so, it is necessary to distinguish the target for modification from that needed for restriction. Restriction is only elicited when two unmethylated target sequences are in inverse orientation with respect to each other and, as shown in **Figure 3(b)**, replication of modified DNA leaves all unmodified targets in the same orientation.

The bifunctional R-M complex is made up of two subunits, the products of the *mod* and *res* genes. The Mod subunit is sufficient for modification, while the Res and Mod subunits together form a complex with both activities (see **Figure 2**). The Mod subunit is functionally equivalent to the MTase (M_2S) of Type I systems and, as in Type I R-M systems, imparts sequence specificity to both activities.

Type IV Restriction Systems

These systems only cut modified DNA, but in contrast to Type IIM enzymes, not within a specific target sequence. They are variable in their complexity and requirements.

E. coli K-12 encodes three distinct, sequence-specific, modification-dependent systems. Mrr is distinguished by its ability to recognize DNA containing either methylated adenine or 5-methylcytosine in the context of particular, but as yet undefined, sequences. McrA and McrBC both restrict DNA-containing modified cytosines (HMC or methylcytosine). The Mcr systems (modified cytosine restriction) are those first recognized by Luria and Human by their ability to restrict nonglucosylated T-even phage (originally called RglA and RglB; restricts glucose-less phage). McrBC is a complex enzyme with a requirement for GTP rather than ATP.

R-M Enzymes as Model Systems

Sequence Recognition, Including Base Flipping

Structures of the crystals of several Type II restriction ENases have been determined – some in both the presence and the absence of DNA. The symmetrically arranged dimers of the Type II enzymes bind to their specific target sequences by the combined effects of different types of interactions including hydrogen bonding and electrostatic interactions of amino acid residues with

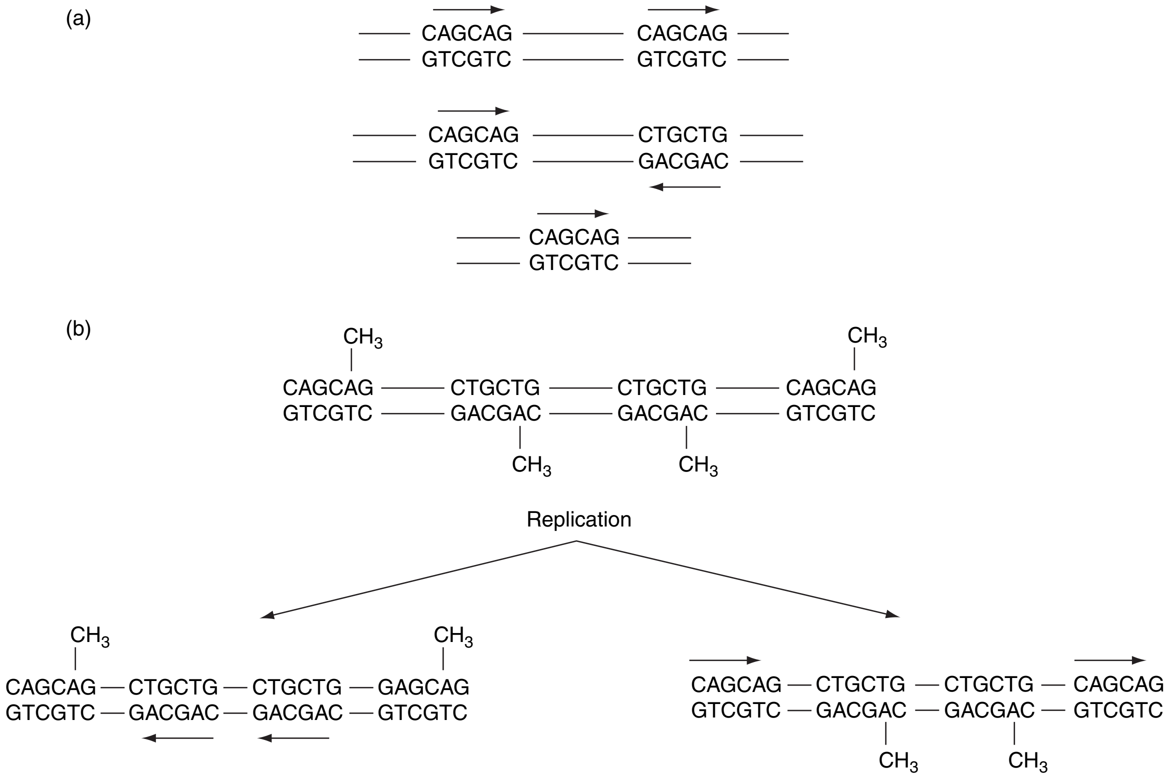
(a)
CAGCAG CAGCAG
GTCGTC GTCGTC
CAGCAG CTGCTG
GTCGTC GACGAC
CAGCAG
GTCGTC
(b)
CH3
CH3
CAGCAG CTGCTG CTGCTG CAGCAG
GTCGTC GACGAC GACGAC GTCGTC
CH3
CH3
Replication
CH3
CH3
CAGCAG — CTGCTG — CTGCTG — GAGCAG
GTCGTC — GACGAC — GACGAC — GTCGTC
CAGCAG — CTGCTG — CTGCTG — CAGCAG
GTCGTC — GACGAC — GACGAC — GTCGTC
CH3
CH3

Figure 3 (a) DNA substrates for a Type III R-M system (EcoP15I). The top strand of each duplex is written 5′ to 3′; the arrows identify the orientation of the target sequences. Solid lines indicate polynucleotide chains of undefined sequence. Only pairs of target sequences shown in inverse orientation (line 2) are substrates for restriction. A single site in any orientation is a target for modification; (b) replication of modified DNA leaves all unmodified target sites in the daughter molecules in the same orientation, and therefore insensitive to restriction.

the bases and the phosphate backbone of the DNA. No general structure, such as a helix-turn-helix or zinc finger (often found in proteins that interact with DNA), is characteristic of the protein–DNA interface, and amino acids that are widely separated in the primary sequence may be involved in interactions with the target nucleotide sequence. Comparisons of the active sites of EcoRV, EcoRI, and PvuII identify a conserved tripeptide sequence in close proximity to the target phosphodiester of the DNA backbone and a conserved acidic dipeptide that may represent the ligands for Mg^{2+}, the catalytic cofactor essential for ENase activity.

The structure of a monomeric MTase interacting with its target sequence identified an important solution to the question of how enzymes that modify a base within a DNA molecule can reach their substrate. The cocrystal structure of M.HhaI bound to its substrate showed that the target cytidine rotates on its sugar–phosphate bonds such that it projects out of the DNA and fits into the catalytic pocket of the enzyme. Such base flipping was confirmed for a second enzyme, M.HaeIII, which also modifies cytosine, and circumstantial evidence supports the notion that this mechanism may be true for all MTases regardless of whether they methylate cytosine or adenine residues.

Comparative analyses of the amino acid sequences of many MTases identified a series of motifs, many of which are common to MTases irrespective of whether the target base is cytosine or adenine. These motifs enable structural predictions to be made about the catalytic site for DNA methylation in complex enzymes for which crystals are not available.

DNA Translocation

Specific interactions of large R-M enzymes with their DNA substrates are not readily amenable to structural analysis. The molecular weight of EcoKI is in excess of 400 000 and useful cocrystals with DNA have not yet been reported. Nevertheless, these complex enzymes have features of mechanistic interest. Much evidence now supports models in which DNA restriction involves the translocation of DNA in an ATP-dependent process prior to the cutting of the substrate. In the case of Type I R-M enzymes the breaks in the DNA may be many kb remote from the target sequence. Molineux and colleagues, in 1999, using assays with phage, have shown that EcoKI can transfer (translocate) the entire genome (39 kb) of phage T7 from its capsid to the bacterial cell.

For linear DNA, the evidence supports the idea that cutting by Type I R-M systems occurs preferentially midway between two target sequences. For Type III enzymes the breaks are close to the target sequence, but in both cases the endonuclease activity may be stimulated by the collision of two translocating protein complexes.

The most conserved features of the polypeptide sequences of Type I and Type III R-M are the so-called DEAD-box motifs, which are also found in RNA and DNA helicases, and the motifs characteristic of adenine MTases. The DEAD-box motifs acquired their collective name because a common variant of one element is Asp-Glu-Ala-Asp, or DEAD when written in a single-letter code. The DEAD-box motifs, which include sequences diagnostic of ATP binding, are found in the subunit that is essential for restriction (HsdR or Res) but not for modification. It is not known how the ATP-dependent activity drives the translocation of DNA, although circumstantial evidence correlates ATPase activity with DNA translocation. Mutations in each DEAD-box motif have been shown to impair the ATPase, translocase and endonuclease activities of a Type I ENase.

Control and Alleviation of Restriction

Control of Gene Expression

The control of restriction activity is critical for survival of bacterial cells. This can be provided by the regulation of gene expression. It may be useful for protecting host DNA in restriction-proficient cells, but it is especially important when R-M genes enter a new host. Experiments show that many R-M genes are readily transferred from one laboratory strain to another. The protection of host DNA against the endonucleolytic activity of a newly acquired restriction system would be achieved if the functional cognate MTase is produced before the restriction enzyme.

Transcriptional regulation of some of the genes encoding Type II systems has been demonstrated. Genes encoding regulatory proteins, referred to as C-proteins for controller proteins, have been identified in some instances. The C-proteins for a number of systems have been shown to activate efficient expression of the restriction gene. When the R-M genes are transferred to a new environment, in the absence of C-protein there is preferential expression of the modification gene, but following the production of the C-protein the consequent generation of restriction-proficient cells.

Representatives of all three types of classical R-M systems have been shown to be equipped with promoters that could permit appropriate transcriptional regulation of the two activities. For complex R-M systems, despite the presence of two promoters, there is no evidence for transcriptional regulation of gene expression. The hetero-oligomeric nature of these systems offers opportunity for the regulation of the R-M activities by the intracellular concentrations of the subunits and the affinities with which different subunits bind to each other. Nevertheless, efficient transmission of the functional R-M genes of some families of Type I systems requires ClpX and ClpP in the recipient cell. Together these polypeptides comprise a protease. The ClpXP complex functions to degrade the HsdR subunit of an active R-M complex before the endonuclease activity has the opportunity to cleave unmodified chromosomal DNA.

Restriction Alleviation

The efficiency with which *E. coli* specifying a Type I R-M system restricts unmodified DNA is influenced by a number of stimuli, all of which share the ability to damage DNA. Induction of the SOS response leads to a decrease in restriction activity, and one consequence of this is a marked reduction of the efficiency with which the bacteria restrict incoming DNA. This alleviation of restriction is usually monitored by following the EOP of phage – unmodified in the case of classical systems or modified in the case of modification-dependent restriction systems. Alleviation of restriction is characteristic of complex systems and can be induced by ultraviolet light, nalidixic acid, 2-aminopurine and the absence of Dam-mediated methylation. The effect can be appreciable and host systems may contribute to more than one pathway of restriction alleviation. Recent experiments have shown that ClpXP is necessary for restriction alleviation of the EcoKI system; therefore there is a connection between the complex mechanisms by which restriction activity is normally controlled and its alleviation in response to DNA damage. Homologous recombination, required for DNA repair, can generate unmodified targets by synthesis of new DNA strands. A normal function of restriction alleviation is to protect the bacterial chromosome from restriction by resident Type I R-M systems when unmodified targets are generated. ClpXP is not relevant to all Type I R-M systems; therefore, alternative mechanisms of alleviation remain to be determined.

Antirestriction Systems

Many phage, and some conjugative plasmids, specify functions that antagonize restriction. An apparent bias of functions that inhibit restriction by Type I R-M systems may reflect the genotype of the classical laboratory strain *E. coli* K-12, a strain with a Type I but no Type II R-M system.

The coliphage T3 and T7 include an 'early' gene, *ocr* or 0.3, the product of which binds Type I R-M enzymes and abolishes both restriction and modification activities. Ocr does not affect Type II systems. The *ocr* gene is expressed before targets in the phage genome are accessible to host restriction enzymes, so that *ocr*$^+$ phage are protected from R-M by Type I systems. The crystal structure of T7 Ocr has shown that this protein mimics the shape and charge of the DNA substrate. Phage T3 Ocr has an additional activity; it hydrolyzes AdoMet, the cofactor essential for both restriction and modification by EcoKI and its relatives. Bacteriophage P1 also protects its DNA from Type I restriction, but the antirestriction function, Dar, does not interfere with modification. The Dar proteins are coinjected with encapsidated DNA, so that any DNA packaged in a P1 head is protected. This allows generalized transduction to occur between strains encoding different Type I R-M systems.

Coliphage T5 has a well-documented system for protection against the Type II system EcoRI. As with the *ocr* systems of T3 and T7, the gene is expressed early when the first part of the phage genome enters the bacterium. This first segment lacks EcoRI targets, whereas the rest of the genome, which enters later, has targets that would be susceptible in the absence of the antirestriction protein.

Some conjugative plasmids of *E. coli*, members of the incompatibility groups I and N, also encode antirestriction functions. They are specified by the *ard* genes located close to the origin of DNA transfer by conjugation, so that they are amongst the first genes to be expressed following DNA transfer. Like the *ocr* proteins of T3 and T7, the protein encoded by *ard* is active against Type I R-M systems.

Bacteriophage λ encodes a very specialized antirestriction function, Ral, which modulates the *in vivo* activity of some Type I R-M systems by enhancing modification and alleviating restriction. The systems influenced by Ral are those that have a modification enzyme with a strong preference for hemimethylated DNA. Unmodified *ral*$^+$ λ DNA is restricted on infection of a restriction-proficient bacterium, because *ral* is not normally expressed before the genome is attacked by the host R-M system, but Ral enhances the modification of those phage that escape restriction. Ral may act by changing the MTase activity of the R-M system to one that is efficient on unmethylated target sequences.

Some phage are made resistant to many types of R-M systems by the presence of glucosylated HMC in their DNA, for example, the *E. coli* T-even phage and the *Shigella* phage DDVI. The glucosylation also identifies phage DNA and allows selective degradation of host DNA by endonucleases specified by the virulent phage. Nonglucosylated T-even phage are resistant to some classical R-M systems because their DNA contains the modified base HMC, but they are sensitive to modification-dependent systems, although T-even phage encode a protein (Arn) that protects superinfecting phage from McrBC restriction. It has been suggested that some phage have evolved to specify DNA that contains HMC, which counteracts classical R-M systems, and that host-encoded modification-dependent endonucleases are a response to this phage adaptation. In this evolutionary story, the glucosylation of HMC would be the latest mechanism that renders T-even phage totally resistant to most R-M systems.

In some cases, a phage genome can tolerate a few targets for certain restriction enzymes. The few EcoRII sites in T3 and T7 DNA are not sensitive to restriction, because this unusual enzyme requires at least two targets in close proximity and the targets in these genomes are not sufficiently close. For the Type III enzymes the orientation of the target sequences is also relevant. Since the target for restriction requires two inversely oriented recognition sequences, the T7 genome remains refractory to EcoP15I because all 36 recognition sequences are in the same orientation. The unidirectional orientation of the target sequences is consistent with selection for a genome that will avoid restriction. Considerable evidence supports the significance of counter-selection of target sequences in phage genomes, in some cases correlating the lack of target sequences for enzymes found in those hosts in which the phage can propagate.

Distribution, Diversity, and Evolution

Distribution

The technical importance of Type II endonucleases in biological sciences has extended their discovery to include enzymes with more than 250 different specificities, while the detection of Type I and Type III R-M systems continued to rely on *in vivo* experiments. More recently, the sequencing of genomes has revealed that R-M systems are almost ubiquitous in the Eubacteria and the Archaea, although identification of homologous sequences does not guarantee the activity of the predicted enzymes. From a survey of 496 genomes (http://rebase.neb.com/rebase/rebase.html), only 35 lack homologues of known R-M systems. Eubacteria without R-M systems generally have small genomes (<2 Mb) and represent organisms that have undergone genetic reduction following a specialized, and intimate, association with a eukaryotic host, for example, *Buchnera aphidicola* (0.62 Mb), which is an endosymbiont of aphids. Many pathogenic bacteria that infect and grow inside eukaryotic cells do not contain R-M systems, possibly because they no longer encounter phage. Examples of important intracellular pathogens devoid of R-M systems include members of the class *Chlamydiae*, and the spirochaete *Treponema pallidum*. In contrast, another pathogenic spirochaete, *Borrelia burgdorferi*, specifies a wide range of plasmid-borne Type II R-M systems, while its single linear chromosome lacks any R-M homologue. Bacteria with small genomes are not necessarily bereft of R-M systems; for example, *Mycoplasma genitalium* (0.58 Mb) specifies a Type I and a Type II system, while the smallest genome from the Archaea, *Nanoarchaeum equitans* (0.49 Mb), contains genes predicted to specify a Type II R-M system. It would appear that, even under the pressures for genome reduction, there is an important selective advantage for cells containing R-M systems.

Type II systems appear to be more prevalent than the other R-M systems in both the Eubacteria and the Archaea. The abundance of Type II enzymes within a single species can be quite dramatic; for example, strains of the ϵ-proteobacterium *Helicobacter pylori* contain homologues for around 20 Type II R-M systems. *H. pylori* is naturally competent in the uptake of DNA and it appears that many of these R-M systems have been acquired by horizontal gene transfer. Different strains share similar complements of Type II homologues, but only a fraction of these coding sequences express functional protein at any one time. Subsets of R-M genes are inactivated by point mutations while others are switched 'on' and 'off' by a mechanism known as slipped-strand mispairing. It is currently unclear why *H. pylori* should accumulate such an arsenal of inactivated R-M systems; however, it is speculated that the ability to switch on new specificities will prevent phage from infecting cells of an entire population.

Enteric bacteria have been used extensively in genetic studies, particularly *E. coli* and *Salmonella enterica*. The evidence from these bacteria is consistent with intraspecific diversity. In *E. coli*, there are at least six distinct mechanistic classes of restriction enzyme, that is, Types I, IIE, IIG, IIP, III, and IV. The Type II systems in *E. coli* currently include about 30 specificities, and at least 14 Type I specificities have been identified.

Diversity and Evolution

R-M enzymes may be dissected into modules. A Type II MTase comprises a TRD and a module that is responsible for catalyzing the transfer of the methyl group from AdoMet to the defined position on the relevant base. The catalytic domains share sequence similarities, and these are most similar when the catalytic reaction is the same, that is, yields the same product (e.g., 5 mC). Given the matching specificities of cognate ENase and MTase, it might be expected that their TRDs would be of similar amino acid sequence. This is not the case; it seems likely that the two enzymes use different strategies to recognize their target sequence. Each subunit of the dimeric ENase needs to recognize one half of the rotationally symmetrical sequence whereas the monomeric MTase must recognize the entire sequence. The absence of similarity between the TRDs of the ENase and its cognate MTase suggests that they may have evolved from different origins.

Restriction enzymes that recognize the same target sequence are referred to as isoschizomers. A simple expectation is that the TRDs of two such enzymes would be very similar. This is not necessarily so. Furthermore the similarities observed do not appear to correlate with taxonomic distance. The amino acid sequences of the isoschizomers HaeIII and NgoPII, which are isolated from bacteria in the same phylum, show little, if any, similarity whereas the isoschizomers FnuDI and NgoPII, which are isolated from bacteria in different phyla, are very similar (59% identity).

Type I R-M systems are complex in composition and cumbersome in their mode of action, but they are well suited for the diversification of sequence specificity. A single subunit (HsdS or S) confers specificity to the entire R-M complex and to the additional smaller complex that is an MTase. Any change in specificity affects restriction and modification concomitantly. Consistent with their potential to evolve new specificities, Type I systems exist as families within which members, for example, EcoKI and EcoBI, are distinguished only by their S subunits. Currently, allelic genes have been identified for at least seven members of one family; each member having a different specificity. It is more surprising to find that allelic genes in *E. coli*, and its relatives, also specify at least two more families of Type I enzymes. While members of a family include only major sequence differences in their S polypeptides, those in different families share very limited sequence identities (usually 18–30%). Clearly, the differences between gene sequences for Type I R-M systems are no indication of the phylogenetic relatedness of the strains that encode them. It is of interest to note that despite the general absence of sequence similarities between members of different families of Type I enzymes, pronounced similarities have been identified for TRDs from different families when they confer the same sequence specificity.

The information from gene sequences for both Type I and Type II systems, as stated by Raleigh and Brooks, in 1998, 'yields a picture of a pool of genes that have circulated with few taxonomic limitations for a very long time'.

Allelic variability is one of the most striking features of Type I R-M systems. Both the bipartite and asymmetrical nature of the target sequence offer more scope for diversity of sequence specificity than the symmetrical recognition sequences of Type II systems. The S subunit of Type I enzymes includes two TRDs, each specifying one component of the target sequence. This organization of domains makes the subunit well suited to the generation of new specificities as the consequences of either new combinations of TRDs or minor changes in the spacing between TRDs. In the first case, recombination merely reassorts the regions specifying the TRDs and, in the second, unequal crossing-over within a short duplicated sequence leads to a change in the spacing between the TRDs. Both of these processes have occurred in the laboratory by chance, as well as by design. The protection of unmodified target sequences in the host chromosome, by restriction alleviation, enhances the opportunity for changes in specificity.

While many bacteria conserve the close linkage of the three genes of Type I R-M systems (*hsdR, hsdM*, and *hsdS*; **Figure 2**), a number of bacteria have been described which contain multiple copies of *hsdS* that are phase-variable. Shuffling the DNA sequences that encode TRDs of different HsdS proteins provides a dynamic method for varying specificity. In *Mycoplasma pulmonis*, there are two examples of 'shufflons', systems that recombine *hsdS* genes. Both shufflons contain *hsdR* and *hsdM* genes flanked by two *hsdS* genes that are inverted with respect to each other. Recombination between these two copies of *hsdS* can generate four different target specificities. A more complex shufflon exists in the genome of the human commensal *Bacteroides fragilis*, which contains an *hsd* locus with the capacity to generate eight HsdS proteins with different specificities.

For Type I R-M systems, the swapping or repositioning of domains can create enzymes with novel specificities, but the evolution of new TRDs with different specificities has not been witnessed. In one experiment strong selection for a change that permitted a degeneracy at one of the seven positions within the target sequence failed to yield mutants with a relaxed specificity.

Biological Significance

The wide distribution and extraordinary diversity of R-M systems, particularly the allelic diversity documented in enteric bacteria, suggest that R-M systems have an important role in bacterial communities. This has traditionally been considered to be protection against phage. Laboratory studies following bacterial populations under conditions of phage infection indicate that R-M systems provide only a transitory advantage to bacteria. Essentially, an R-M system with a different specificity could assist bacteria in the colonization of a new habitat in which phage are present, but this advantage would be short lived as phage that escape restriction acquire the new protective modification and bacteria acquire mutations conferring resistance to the infecting phage. It can be argued that one R-M system protects against a variety of phage, and the maintenance of one R-M system may compromise the fitness of the bacterium less than the multiple mutations required to confer resistance to a variety of phage – especially because these are likely to occur in important components of the cell, such as surface receptors. No direct evidence supports this expectation. It may be relevant to remember that the restriction barrier is generally incomplete, irrespective of the mechanism of DNA transfer, and that the fate of phage and bacterial DNA fragmented by ENases may differ. A single cut in a phage genome is sufficient to prevent infectivity. Fragments generated from bacterial DNA will generally share homology with the host chromosome and could be rescued by recombination. The rescue of viable phage by homologous recombination requires infection by more than one phage or recombination with phage genomes that reside within the host chromosome.

A protective role for R-M systems in no way excludes an additional one that is relevant to bacterial evolution. Restriction provides an opportunity to separate linked genes while concomitantly generating DNA breaks that can facilitate gene transfer simply by ligation, but primarily by general recombination pathways. In *E. coli*, and probably bacteria in general, linear DNA fragments are vulnerable to degradation by exonucleases, particularly ExoV alias RecBCD. The products of restriction, therefore, are substrates for degradation by the very enzyme that is an essential component of the major recombination pathway in *E. coli*. However, degradation by RecBCD is impeded by the special sequences, designated Chi, which stimulate recombination. It has been shown that a Chi sequence can stimulate recombination when RecBCD enters a DNA molecule at the site generated by cutting with EcoRI. It seems inevitable that fragmentation of DNA by restriction would reduce the opportunity for recombination to incorporate long stretches of DNA, but given that DNA ends are recombinogenic, restriction could promote the acquisition of short segments of DNA.

Radman and colleagues, in 1989, suggested that R-M systems are not required as interspecific barriers to recombination, since the DNA sequence differences between *E. coli* and *Salmonella* are themselves sufficient to hinder recombination. It is evident, however, that selection has maintained a diversity of restriction specificities within one species, and consequently restriction is presumed to play a significant role within a species, where DNA sequence differences are less likely to affect recombination. Analyses of the effects of restriction on the transfer of DNA between strains of *E. coli* by transduction have shown that restriction diminishes the size, and alters the distribution, of DNA segments acquired by homologous recombination.

Kobayashi and colleagues have viewed R-M genes as 'selfish' entities on the grounds that loss of the plasmid that encodes them leads to cell death. The experimental evidence for some Type II R-M systems implies that the cells die because residual ENase activity cuts incompletely modified chromosomal DNA. The behavior of Type I systems, on the other hand, is different and is consistent with their ability to diversify sequence specificity; new specificities are acquired by recombination, and old ones are readily lost without impairing cell viability.

Applications and Commercial Relevance

Initially, the opportunity to use enzymes that cut DNA molecules within specific nucleotide sequences added a new dimension to the physical analysis of small genomes. At the beginning of the 1970s, maps (restriction maps) could be made in which restriction targets were charted within viral genomes and their mutant derivatives. Within a few years the same approach was generally applicable to larger genomes. The general extension of molecular methods to eukaryotic genomes depended on the technology that enabled the cloning of DNA fragments, that is, the generation of a population of identical copies of a DNA fragment. In short, DNA from any source could be broken into discrete fragments by restriction ENases, the fragments could be linked together covalently by the enzymatic activity of DNA ligase, and the resulting new combinations of DNA amplified following their recovery in *E. coli.* Of course, to achieve amplification of a DNA fragment, and hence a molecular clone, it was necessary to link the DNA fragment to a special DNA molecule capable of autonomous replication in a bacterial cell. This molecule, the vector, may be a plasmid or a virus. Importantly, it is usual for only one recombinant molecule to be amplified within a single bacterial cell. In principle, therefore, one gene can be separated even from the many thousands of other genes present in a eukaryotic cell, and this gene can be isolated, amplified and purified for analysis. The efficiency and power of molecular cloning have evolved quickly, and the new opportunities have catalyzed the rapid development of associated techniques, most notably those for determining the nucleotide sequences of DNA molecules, the chemical synthesis of DNA and, more recently, the extraordinarily efficient amplification of gene sequences *in vitro* by the polymerase chain reaction (PCR). In some cases, amplification *in vitro* now obviates the need for amplification *in vivo* since the nucleotide sequence of PCR products can be obtained directly.

The bacterium *E. coli* remains the usual host for the recovery, manipulation, and amplification of recombinant DNA molecules. Already, however, for many of the commonly used experimental organisms the consequence of a mutation can be determined by returning a manipulated gene to the chromosome of the species of origin.

The recombinant DNA technology, including screens based on the detection of DNA by hybridization to a specific probe and the analysis of DNA sequence, is now basic to all fields of biology, biochemistry, and medical research, as well as the 'biotech industry'. Tests dependent on DNA are used to identify contaminants in food, parents of children, persons at the scene of a crime, and the putative position of a specimen in a phylogenetic tree. Mutations in specific genes may be made, their nature confirmed, and their effects monitored. Gene products may be amplified for study and use as experimental or medical reagents. Hormones, cytokines, blood-clotting factors, and vaccines are among the medically relevant proteins that have been produced in microorganisms, obviating the need to isolate them from animal tissues.

Most of the enzymes used as reagents in the laboratory are readily available because the genes specifying them have been cloned in vectors designed to increase gene expression. This is true for the ENases used to cut DNA. It is amusing to remember that in the 1980s the generally forgotten, nonclassical, restriction systems identified by Luria and Human, in 1952, were rediscovered when difficulties were encountered in cloning Type II R-M genes. It was soon appreciated that cloning the genes for particular MTases was a problem in 'wild-type' *E. coli* K-12; the transformed bacteria were killed when modification of their DNA made this DNA a target for the resident Mcr (Type IV) restriction systems. Rare survivors were *mcr* mutants ideal strains for recovering clones of foreign DNA rich in 5 mC as well as genes encoding MTases.

Further Reading

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Relevant Website

http://rebase.neb.com/rebase/rebase.html – REBASE^R The Restriction Enzyme Database

DNA Sequencing and Genomics

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Glossary

assembly The process by which small sections of DNA sequence generated by various sequencing processes are compiled to recreate the original chromosome from which the DNA originated.

contigs The DNA sequence reconstructed from a set of overlapping DNA segments through the assembly process.

genome An organism's genetic material, including ancillary plasmids, and chromosomes in the case of microbes.

genomics The study of an organism's entire genome.

next-generation sequencing (also Next-Gen sequencing) A catchphrase used to describe the recently developed sequencing platforms that employ non-Sanger methodologies to derive DNA sequences.

scaffolds A series of contigs that are consecutively ordered but not necessarily continuously connected.

Abbreviations

ABI	Applied Biosystems
APS	adenosine phosphosulfate
ATP	adenosine triphosphate
CCD	charge coupled device
COBRA	constraint-based reconstruction and analysis
PCR	polymerase chain reaction
TIGR	The Institute of Genome Research
TIRM	total internal reflection microscopy

Defining Statement

Microbial genomics has expanded rapidly since the 1995 sequencing of *Haemophilus influenza*. This article will examine the influential role genomics has played in many fields, and how technological advances enabled the sequencing of the first genomes. The impact of next-generation, or Next-Gen, sequencers on genomics and their role in enabling novel applications will also be discussed.

Introduction

Understanding of microbial biology has been greatly enhanced by the introduction of genomic sequence data. *Haemophilus influenza* was the first microbe sequenced in 1995, followed by *Saccharomyces cerevisiae* in 1996 and *Escherichia coli* in 1997. The number and rate of completed microbial genomes increased dramatically over the following years (**Figure 1**), with the sequences of over 2000 strains of *H. influenza* available to the scientific community by February 2007. Sequencing has been completed for 709 different microbial genomes and an additional 98 genomes are in draft as of May 2007.

As the number of completed microbial genome sequences has increased, so too has both the size of the genomes, from 1.83 Mb for *H. influenza* to 87 Mb for *Gibberella moniliformis*, and the complexity of samples sequenced, from single organisms to entire microbial communities sampled from diverse environments such as acidic mines, the open ocean, or the human gut. In light of this considerable expenditure of time and money, it is only reasonable to ask what benefit is obtained from these microbial sequencing projects.

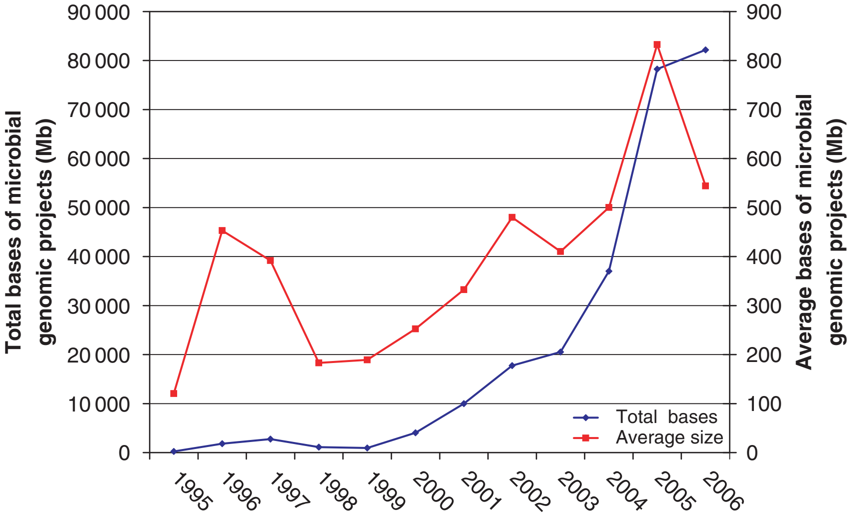

Figure 1 Increasing amount and size of microbial genomic sequencing. Both the total amount of sequence generated (blue) and the average size of the microbial genome sequenced (red) exhibit an upward trend since 1995. Raw data were obtained from GOLD Genomes OnLine Database V2.0 (www.genomesonline.org).

Benefits of Microbial Genomics

The principal goal of sequencing microbial genomes is directly related to human health. Although a vanishingly small proportion of existing bacterial species are clinically relevant, approximately 47% of the bacterial species that have been sequenced, or are scheduled for sequencing, are important human pathogens. Possession of the complete genome sequence of medically important microbes has obvious implications in many areas pertaining to human health. Sequence information can be used to generate either polymerase chain reaction (PCR)- or microarray-based tests that can specifically detect and classify pathogenic microbes, enabling discrimination of closely related species more precisely than culture-based tests. This is particularly evident with regard to detecting and classifying species within the *Bacillus cereus* bacterial group. This group contains bacteria that range in impact from potential bioterrorist threats (*Bacillus anthracis*) to the source of the most commonly utilized biological pesticide in the world (*Bacillus thuringiensis*). Given the strain- and species-specific breadth of health impact and the required response, accurate classification and detection are essential. In the case of *B. anthracis*, conventional species identification includes a variety of microbiological and biochemical assays including culturing and animal testing. These tests typically require several days to complete, and can produce false-positive results owing to the high degree of genetic and phenotypic similarities found between *B. anthracis* and *B. cereus* cultures.

Diagnostics

In recent years diagnostic advances in terms of both speed and accuracy have been achieved using genetic information to design molecular tools including both standard and real-time PCR- and microarray-based tests, typically targeting the virulence-inducing pX01 and pX02 plasmids, and enabled by the possession of the complete sequence of both plasmids. Complete diagnostic reliance upon markers from these two plasmids is risky, however, as horizontal gene transfer and/or plasmid loss can confound discrimination between *B. cereus* species. More recent diagnostic platforms utilize multiple markers, generating microarrays with the ability to accurately discriminate *B. anthracis* from the other species in the *B. cereus* group. Multiple markers also allow the detection and quantification of multiple species of bacteria within a complex population background, which is often difficult or impossible to achieve using conventional culturing techniques. Clearly, nucleic acid sequences are required for the design of appropriate PCR primers and probes and are obtained through extensive sequencing of target microbial genomes at sufficient depth to determine variable and conserved regions at both inter- and intraspecific levels. Probe sets have also been designed and utilized to rapidly detect and identify disease-causing microorganisms and have found a routine application in monitoring the safety of food and water supplies. The technique has evolved to the point where in the case of a *Campylobacter jejuni* outbreak, bacteria from different hosts (livestock, chicken, human, and environmental) had distinct genetic fingerprints that permitted outbreaks to be traced back to the original source of the infection.

Detailed information on inter- and intraspecific genetic variability has also proven essential in identifying mutations that confer antibiotic resistance thus indicating antibiotics to which microbes may be immune. By correctly identifying both the pathogen and the proper antibiotics, the speed and effectiveness of therapeutic regimens can be optimized.

Therapeutics

Genomic information also enhances the suite of therapeutic options available to the medical community. In response to the ready availability of genomic data, the process of drug development has changed from searching for drugs active against specific cells to those active against particular proteins. Establishment of large microbial sequence databases has allowed identification of organism-specific genes. These genes are then screened by a variety of methods to determine which of the genes are essential for microbial growth and are not common genes in eukaryotic pathways, thus decreasing the risk of side effects. Essential genes, conserved within the particular microbe, are prime targets for drugs designed to disrupt or inhibit the essential gene activity within the microbe. Using this technique the first new tuberculosis-specific drug in 40 years was discovered. The genomes of mutant strains of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* resistant to the drug DARQ R207910 were compared with the sequence from wild-type, nonresistant strain of *M. tuberculosis*, thereby identifying both the mutations responsible for resistance and the drug target, the proton pump of adenosine triphosphate (ATP) synthase. The theory that these drugs might act with greater specificity and lower side effects than drugs that target entire cells or common metabolic processes (which are more likely to be shared with the host) has been supported by high human tolerance in clinical trials.

Similarly, microbial bioinformatics can be used to streamline vaccine development through a process known as reverse vaccinology. As opposed to traditional vaccines that rely upon exposure to dead or attenuated pathogens, this process screens microbial sequence databases to detect pathogen-specific proteins (typically membrane-associated, virulence-related, or secreted) that could induce a protective immune response. The technique was initially demonstrated by reducing 570 coding genes to 7 surface-expressed proteins with potential as vaccine candidates, and is now being applied to many pathogens.

Pathogenicity

Intensive research on microbial genomes also produced a deeper understanding of the multiple mechanisms of pathogenicity itself, whether arising within a species through nucleotide mutation, or passed from one species to another through gene transfer. Of particular interest to medicinal genomics are pathogenicity islands, a pathogenic gene-containing subclass of genomic islands, large chromosomal or plasmid-based clusters of functionally related genes flanked by repeat sequences. The identification of pathogenicity islands provides an important medical tool to detect and track the movement of pathogenic activity within and between bacteria species. Additionally, the homology to known pathogenic island sequences permitted identification of other putative virulence factors in microbial sequence databases.

Genetics and Evolution

Extensive studies of pathogenicity islands, driven by their relevance to human health, also had a dramatic impact on the conventional view of microbial genetics, ecology, and evolution. Comparison of whole-genome sequences has led to the identification of genomic islands, a large class of genetic elements that differ in GC content from the host genome, are flanked by repeat sequences, and are typically located near a tRNA and either an integrase or a transposase gene. The functions of the genes contained within the genomic islands vary widely, and subdivide genomic islands into pathogenicity, antibiotic, secretory system, metabolic, and symbiosis islands. As the transfer mechanism of pathogenicity islands was illuminated, the prevailing view of microbial evolution as a process driven by small-scale shifts of nucleotide mutations gave way to one that often occurred in large quantum leaps. This view envisions microbial evolution as driven by the horizontal transfer of genomic islands from one bacterium to another, ignoring species boundaries, even to the point where gene transfer has been shown to occur between archaea and bacteria. This horizontal transfer of genes between bacteria is a common, universal evolutionary event, although the amount of horizontal gene transfer evident in any given species varies considerably. With the incorporation of foreign genes, the host genome is modified, and new phenotypes may emerge, even resulting in speciation. Interestingly, not all cases of lateral gene transfer occur within bacterial species; recent research has revealed instances of gene transfer between bacteria and eukaryotes. Most importantly, the ubiquitous nature of genomic islands in microbial genomes has reinforced the view of dynamic bacterial genomes, where a constant, species-specific genetic backbone or core genome is variably augmented with genomic inserts such that the presence of a specific genomic island in one strain does not indicate the presence of the same island in another strain of the same species.

Minimum Genome Size

The abundance of available microbial sequences has highlighted wide differences in the size of microbial genomes, ranging from less than 500 kb to over 50 Mb. This observation has raised questions regarding the process through which genes can be lost and the minimum genome size required to sustain life. Early work using mutational studies suggested a minimum viable genome size of 318 kb, which under the assumption of 1.25 kb per gene, estimates that 250 genes are required for life. Comparison of *Mycoplasma genitalium* and *H. influenzae* genes arrived at a similar estimate of 256 essential genes, in close agreement with the estimate of 265–350 minimal genes obtained by mutagenesis of the *Mycoplasma* genome. While this research might initially appear to be a strictly academic pursuit, answers to these questions have broad implications for bioengineering and the emerging field of synthetic biology. Once the requisite genes for life are known, opportunities exist to modify existing organisms to produce desired behavior or products, or to design artificial life forms from scratch, employing genomes containing as few as 151 genes. Sequencing technology and capacity have expanded to the point where researchers now initiate projects that intend to sequence genomes from whole ecosystems rather than from whole organisms.

Metagenomics

Metagenomics is an inclusive term that encompasses a variety of techniques pioneered that use extracted bulk nucleic acid taken directly from a given ecosystem, and fragment and sequence it, as opposed to culturing target microbes from the environmental samples under laboratory conditions and subsequently sequencing the purified cultures. The main benefit of the metagenomic approach is the obviation of laboratory cultures, enabling the sequencing of microbes unculturable under normal conditions, such as extremophiles, thermophiles, and symbionts. Inclusion of unculturable organisms in community profiles is essential for accuracy; as many as 99% of microbes may be unculturable and hence invisible to community profiles developed with traditional culture and sequence technologies. The goal of metagenomic studies is different from that of traditional organismal sequencing; metagenomics typically does not seek to complete each of the component genomes but rather seeks to sequence enough of each organism to identify the component organisms and their relative abundance in the sample. In addition, the unassembled sequences are used for quantitative gene content analysis, which utilizes the information from the metabolic genes detected in the community to derive information about the habitat. Metagenomic projects have been used to survey the population composition from samples as diverse as seawater, acid mines, the distal gut of mice and humans, as well as whale carcasses and agricultural soil. The difficulty with metagenomics projects stems from the sheer volume of data produced, the amount of sequencing required to generate it, the computational power required to analyze it, and the size of the existing database of known sequences necessary to classify the results. For example, close to 120 000 rRNA PCR amplicons were sequenced for one of the marine studies, and target phylogeny was classified by matching the sequences against a database containing a similar number of published rRNA sequences. Another seawater survey sequenced, annotated, and classified more than 1×10^9 bases of a nonredundant sequence from 1.66 million individual sequences in their characterization of microbial communities. As the size of the sequence databases increases, so will the accuracy with which metagenomic studies identify community composition, but with a concomitant increase in the required computing power.

An additional, but by no means exhaustive, list of the benefits accrued from extensive microbial sequencing includes the following: genomic databases provide essential information for the development of alternative technologies, enabling the design of oligonucleotide primers and probes for PCR- and microarray-based applications, and repositories of completed genomes serve as the foundation for subsequent genome sequencing, facilitating gene annotation, defining gene coordinates, identifying putative orthologues, paralogues, and homologues; providing the basis for discovery of novel diagnostic or therapeutic polymorphisms; and supplying the reference data required to identify microbes from fragments of sequenced nucleic acids. Also, completed whole genomes permit the creation of genomic-scale metabolic models that direct the bioengineering of industrially or medically useful behaviors or products for such diverse fields as development of alternative energy sources (such as ethanol, methane, or hydrogen), bioremediation of toxic chemicals, energy generation, and production of enzymatic or chemical catalysts.

Sequencing Technology

The demand for microbial sequencing has been driven by the increased utility and relevance of the data, but meeting this demand has been enabled by evolution and optimization of the sequencing process itself, providing ever-increasing amounts of sequence data per unit time at a lower cost with every passing year as is clear from **Figure 2**.

DNA sequencing as we currently know it started in the mid-1970s with the development of two gel electrophoresis-based sequencing systems, one designed by Sanger and Coulson and the other by Maxam and Gilbert. These two methods supplanted the previous process, which relied upon fragmentation of the nucleic acids through various methods, followed by chromatographic resolution of the fragments, with subsequent identification of the sequence by base-specific size shifts after electrophoresis or analysis of the terminal nucleotide.

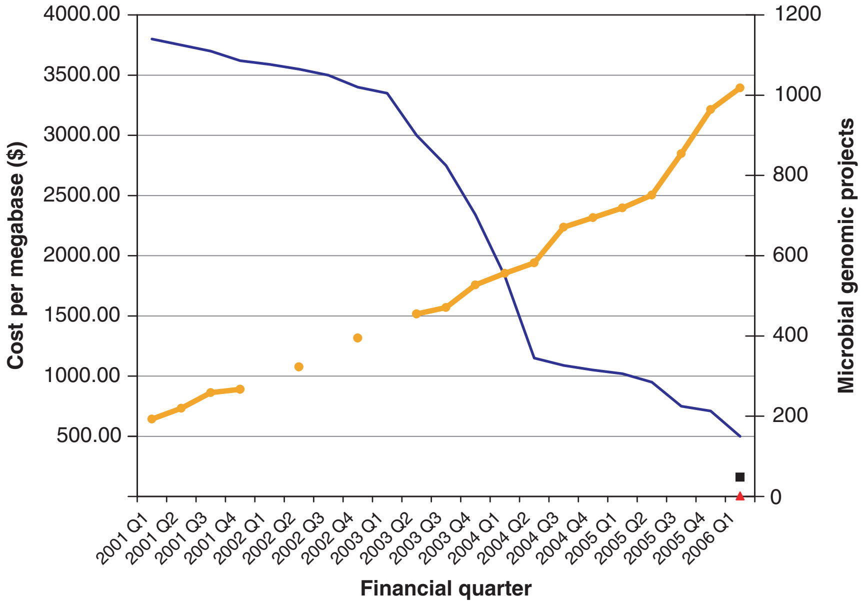

Figure 2 Inverse relationship between amount and cost of sequencing. The number of microbial genomic sequencing projects (orange line) increases by quarter as the cost per megabase of sequencing using capillary array electrophoresis (blue line) at a major sequencing center decreases. For reference, cost per megabase is also shown for the 454 Life Sciences GS-20 ($166, black square) and the Solexa 1G ($5, red triangle). Raw data for genomic projects were obtained from GOLD Genomes OnLine Database V2.0 (www.genomesonline.org).

The Sanger Method

Despite their similarity in the utilization of gel electrophoresis to size fractionate bands of ending at every possible base in the sequence of interest, the Sanger and Maxam–Gilbert strategies differed significantly in methodology. The Sanger method relied upon two serial polymerase extension reactions from a distinct priming site, the first of which generated partial fragments of every possible length from the initial priming site along the template, producing a population of fragments, some portions of which were terminated at each of the nucleotides in the target sequence. The product of this initial reaction was then split into eight aliquots that were then used as primer for the second round of amplification. In four of the reactions, the reaction mix contained all but one of the four nucleotides, with a different nucleotide absent in each of the four reactions. In the other four reactions, only one nucleotide was added, with a different nucleotide present in each mix. In the first four reactions, the primer would be extended until the nucleotide complementary to the missing base was encountered, at which time the amplification process would halt for that strand. In the second reactions, the variable length primers would elongate only as far as the template contained bases complementary to the single nucleotide included in the reaction mix. As a radioactive ^{32}P nucleotide had been incorporated in each product during the initial amplification step, the products of all the eight reactions could be visualized on a gel after exposure on X-ray film. The sequence was determined by counting the individual bands in each lane on the exposed film; the main disadvantage with this system was the difficulty in determining the length of homopolymer tracts, as bands were generated for only the first and the last nucleotide, and the length had to be inferred from the gap between the two. Nonetheless, the system was initially capable of generating approximately 50 base reads and was used to sequence the entire 5368 base pair viral φX genome.

The Maxam–Gilbert Method

The Maxam–Gilbert method, in contrast, enzymatically cleaved the double-stranded template. The 5′ ends of the resulting fragments were then labeled with ^{32}P. These radiolabeled fragments where then aliquotted into four reactions: one fragmented by a chemical reaction that attacked only C nucleotides; another attacked only G nucleotides; a third cleaved A, with some cleavage of G nucleotides; and the fourth predominantly cleaved T nucleotides, with some cleavage at C sites. Like the Sanger method, the sequencing process was resolved by electrophoresis and photographic exposure. The Maxam–Gilbert method, however, was able to resolve homopolymer regions accurately with sequentially sized bands indicating all bases in the tract, leading to an initial dominance of this method.

Improved Sanger

The Sanger method was later modified to include dideoxy terminators, an improvement that resolved the outstanding issue of homopolymers. Then the process, also known as the 'chain termination method', utilized nucleotide analogues for all four of the normal deoxynucleoside triphosphates. Sequencing was conducted in four reactions with each containing all dNTPs; but in each of the reactions a different nucleotide was present in a reduced amount, and the analogue for that particular nucleotide was added as well. As polymerases began to copy the template from the initial priming site, inclusion of the analogue would result in termination of the nascent strand. As the analogues were present in reduced concentration, incorporation was an unlikely event for any single strand, but a termination was statistically likely to occur at each nucleotide position in the sequence when the entire reaction mix was considered. Now each of the bases in a given homopolymer region generated a specific band, and could be accurately sequenced. This, in conjunction with the subsequent inclusion of fluorescently labeled terminators, a shorter reaction protocol, the absence of toxic chemicals, and the ease of optimization for automated sequencing resulted in the widespread acceptance of the Sanger method and its continued relevance to genomics.

Automated Sequencing

Automation of DNA sequencing became possible once fragment detection was conducted via fluorescence rather than radiation. The development of sequencing primers possessing 5′ fluorescent labels with distinct fluors for each nucleotide increased throughput by allowing all four nucleotide-specific reactions to be run in a single gel lane, and also enabled automated gel resolution by employing lasers, rather than film exposure, for base detection. Commercial automated slab-gel sequencers were first used in 1987; the ABI 370A, which had ran 16 lanes per plate, generated roughly 400 bases per template, for a total yield of ~6500 bases per 14-h run. The throughput for slab-gel systems improved as the number of lanes that could be simultaneously sequenced increased to 96, with as many as 200 lanes sequenced on proof-of-concept devices. Additional advantages were realized as the thickness of the gel decreased from 400 to 50 μm, permitting the use of higher electrical field strengths during electrophoresis and reducing heat retention, thermal distortion, and temperature gradients through efficient heat transfer, resulting in gained sequencing speed (from several hours to between 15 and 60 min for reads of a few hundred bases), sensitivity, and resolution, while simultaneously increasing the total output per run by extending read lengths from 100 to 400 bases. The labor invested in sequencing was further reduced on both the front and the back ends of the sequencing process; to address bottlenecks in sample preparation, researchers adapted various robotic work stations and liquid-handling systems to generate templates for sequencing, preparing up to 96 templates simultaneously. After the sequencing run was completed, automated software incorporated the onerous tasks of data acquisition and basecalling, allowing data analysis to keep pace with the improved sequencing throughput.

Capillary Sequencing

The drive for faster sequencing runs and increased automation eventually led researchers from slab-gel based sequencing to systems that utilized electrophoretic capillaries filled with a viscous polymer mix that could resolve sequences up to 14 times faster than traditional gels. Not only did the small-bore capillaries efficiently dissipate heat, allowing high voltage application and rapid run times, but they also provided higher resolution and longer read lengths than the previous slab systems. The capillary-based systems generally proved more efficient in other ways as well: by utilizing polymer-filled capillaries, the lengthy process of gel pouring was obviated, and the fact that samples could be electrokinetically loaded into the capillary from the wells of a 96-well plate was far easier and more user-friendly than the pipette loading required for the slab-gel combs and reduced turnaround time between sequencing runs. The first commercial capillary sequencer was released in the United States in 1995 and possessed only a single capillary that was used to sequence multiple templates in series, fed from a 96-well plate. Potential throughput leaped yet again when it was replaced 3 years later by another capillary unit, this one featuring a 96-capillary array, permitting simultaneous sequencing of 96 samples.

Enzymatic Improvements

Several other advances had major impacts on the amount and quality of microbial sequences entering the database, and many of these were biological and computational rather than mechanical. The first was the continued optimization of the polymerases and other enzymes that formed the core of the sequencing process. Early improvements to the T7 DNA polymerase removed the 3′ to 5′ exonuclease activity and increased the efficiency with which it incorporated nucleotide analogues. This resulted in higher gel resolution and longer read lengths owing to a reduction in terminations at pause sites or sites with challenging secondary-structure motifs. Meticulous optimization of the polymerase reaction conditions resulted in the substitution of Mn^{2+} for Mg^{2+}, addressing an existing problem where polymerases significantly discriminated against dideoxynucleotides, displaying preferential incorporation of deoxynucleotides.

When Mn^{2+} was used, T7 DNA polymerase incorporated dideoxynucleotides and deoxynucleotides at approximately the same rate.

Another improvement involved the addition of the enzyme pyrophosphatase, which prevented pyrophosphorylsis, or polymerase-driven 3′ to 5′ degradation of DNA fragments triggered by high concentrations of pyrophosphate, a by-product of polymerization. In addition, whereas pyrophosphate has been shown to accumulate during amplification and inhibit polymerase activity at high levels, use of pyrophosphatase has been shown to reduce inhibition and ensure high levels of amplification. The combination of modified enzymes and reaction conditions had the net effect of increasing both the length and the quality of the sequences produced in a given run, and thus increased the amount of usable sequence generated.

Sample Preparation

Despite technological advancements in the number and length of reads produced while sequencing, proper orientation of the reads to form the completed template remained one of the serious hurdles that challenged the analysis of genomes or other large sections of DNA. Complete coverage of a genome was facilitated by longer and more plentiful sequence reads, but prior to the incorporation of large-scale computational resources for genome assembly, simply placing the generated sequences in the proper genomic context was difficult. To that end, most of the sample preparation processes at the time initiated the sequencing process from known priming sites within the genome and expanded the coverage sequentially outward from those anchoring points. One such technique, unidirectional deletions, generated an ordered set of subclones by progressively deleting sections from the original template through the activity of a 5′ exonuclease; these increasingly digested fragments were then cloned and sequenced. The primer walking method sequenced fragments generated from a known priming site and used the resulting sequence information to design the primer set necessary to generate the subsequent section of DNA to be sequenced. In this manner, the entire region was sequenced using the data from the preceding fragment to generate primers for the next. Bacterial transposons were employed in the transposon insertion method, where transposons were inserted directly into the fragmented and subcloned template DNA. As the transposon sequences were known, their locations within the subclones could be mapped and could serve as priming sites for directional sequencing.

Whole-genome sequencing received a critical improvement in the optimization of the shotgun sequencing process used in the sequencing of the *H. influenza* genome in 1995. The original technique had been in routine use since 1982 when Fredrick Sanger and his colleagues employed it to reduce a long section of DNA template (in this case the lambda phage genome) into the relatively small fragments of DNA required for successful chain terminator sequencing. Like the deletional and transposon methods, shotgun sequencing relied upon the physical fractionation of the target genome or gene into ordered, overlapping segments, each containing up to 40 kb of DNA. Unlike the other methods, however, each of these large subsections was then shotgunned, or randomly fragmented, amplified through cloning in *E. coli*, and sequenced. A computer program was then used to align all matching sequences from the pool of reads, tying together overlapping sequences and creating a consensus sequence of the original template (known as contigs). In this manner, the short random sequences were assembled back into the larger sections, which in turn were assembled into a complete genome. For an illustration of sample preparation methodologies for whole-genome sequencing refer to **Figure 3**.

Data Analysis and Assembly

Shotgun sequencing, as used for the *H. influenza* project, however, was modified to allow whole genomes to be sequenced completely. The group from The Institute of Genome Research (TIGR) revised the Sanger shotgun method by utilizing double-stranded, randomly fragmented DNA as the template for cloning, as opposed to the single-stranded template used previously. The use of double-stranded template DNA facilitated paired-end sequencing, sequencing from both ends of the cloned fragment, providing sequence information on the fragment's starting and ending regions. This information, combined with the fact that the randomly fragmented templates had been carefully size-selected prior to sequencing, ensured that not only were the ends of the fragments known, but the length of unknown sequence separating them was known as well. This was an important advantage as it permitted the generation of scaffolds, which resulted when one had two or more contigs for which the sequences were completely known, but had no overlapping sequence between the contigs to indicate the order or orientation of the contigs in the broader genomic context. With the modified shotgun sequencing and paired-end sequencing, however, reads that spanned contigs with sequences from either end of the fragment aligning with regions in different contigs could be used to orient the two contigs and to efficiently estimate the amount of missing sequences between them. With this information, PCR primers could be designed to specifically amplify and sequence the missing regions, filling the gaps and completing the genome coverage.

The 24 000 sequencing reads generated during the whole-genome shotgun sequencing of *H. influenza* dwarfed anything that had been analyzed before and

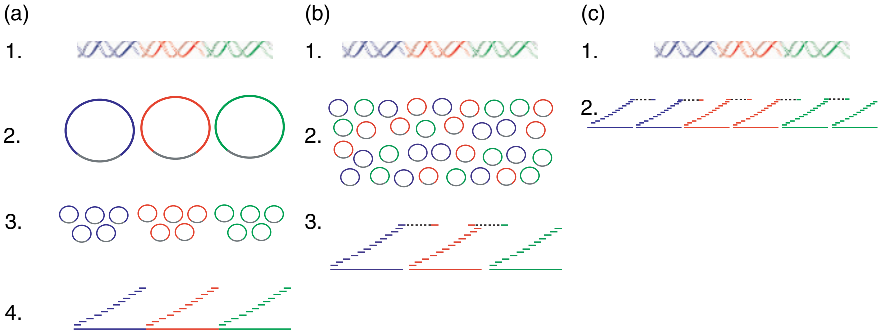

Figure 3 Sample preparation methodologies for whole-genome sequencing. In traditional shotgun sequencing (a), the genome (a1) is fragmented in an ordered fashion and cloned (a2). These clones are then randomly fragmented into sections small enough to be sequenced, and the subsections are cloned and sequenced (a3). The resulting sequence reads from each clone are then overlapped and reassembled to build contigs; the contigs are joined together to compile the genome (a4). In whole-genome shotgun sequencing (b), the entire genome (b1) is randomly fragmented into sequenceable sections (b2), cloned, and sequenced. Extensive computer assembly is then required to reassemble the entire genome from the reads (b3). Paired-end reads (colored blocks linked by dotted lines) are used to orient contigs within the genomic context, forming scaffolds. In next-generation sample preparation (c), clonal amplification obviates the use of cloning, so the genome (c1) is fragmented, amplified, and sequenced. The short reads (25–200 bases) generated are then used to compile contigs (c2), some of which may not completely overlap to form contiguous sections of DNA (note gaps between contigs in c2). As with whole-genome sequencing, paired-end reads (colored blocks linked by dotted lines) are used to orient contigs, but due to the shorter reads, greater reliance is placed on them for reassembly.

overwhelmed any of the existing assembly packages of the day. Alignment and assembly was enabled by the development of TIGR Assembler, a computer program designed specifically to handle the increased data flow. Additional improvements to genomics software and algorithms have increased the information that can be derived from the assembled sequences, particularly in regard to gene annotation, and the development of genome-scale metabolic models of the sequenced organism. One such program, the constraint-based reconstruction and analysis (COBRA) modeling technique, helped develop metabolic models for a variety of organelles and microbes, enabling scientists to assess and modify genome contents, a valuable capability for bioengineering.

These *in silico* improvements allowed the sequencing community to cope with the throughput increases that resulted from regular updates and modifications to the line of capillary array sequencers. It must be realized, however, that most of the microbial genomes sequenced to date are not the products of individual sequencers operated in individual laboratories. Rather, large-scale genomic sequencing typically requires a truly industrialized, automated approach to sequencing, where economies of scale result in multiple colony pickers, fleets of PCR machines, and banks of capillary array sequencers (often 100 or more), all of which operate 24 h a day, every day of the year. After the actual sequencing is completed, massive computer networks are employed to assemble and annotate the sequence. Relatively few institutions are capable of supporting this level of investment, but without this infrastructure whole-genome sequencing has, until now, been too costly and time-consuming for typical laboratories to undertake.

Novel Sequencing Technologies

The current development and utilization of the next-generation, or Next-Gen, sequencers in genomics research provide core facilities, if not individual laboratories, with the ability to complete microbial genomics projects on their own. All of these emerging technologies employ some type of non-Sanger sequencing, and each possesses the potential to truly democratize sequencing, providing individual laboratories with daily sequencing capacities that rival those previously found only at large genome centers (**Figure 4**).

454 Life Sciences

The first of the commercially available next-generation sequencers was the GS-20 released by 454 Life Sciences Corp., Branford, CT, in 2005. Initial publications on the new system detailed the complete sequencing of *M. genitalium* at 99.96% accuracy in less than 5 h, 100 times faster than with conventional sequencing technology. The GS-20 generates between 20 and 35 megabases of sequence per 5.5-h run from 200 000 individual 100–120 base pair reads, employing a miniaturized sequencing process based on pyrophosphate-based sequencing. The GS-FLX, the second-generation 454 sequencer released in 2007, generates over 400 000 individual reads with lengths in the range of 200–300 bases at single read accuracies greater than 99.5%, generating a total of 100 Mb of sequence in less than 8 h.

Unlike Sanger or Maxam–Gilbert sequencing, pyrophosphate-based sequencing utilizes the act of DNA replication by the polymerase to establish the

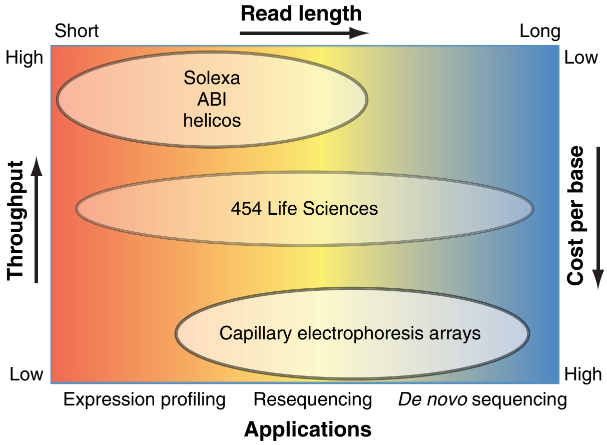

Figure 4 Placement of sequencing technologies within an application matrix. The application matrix uses read length, cost, and throughput to illustrate how the traditional capillary sequencing (long reads, low throughput and high cost per base), 454 Life Sciences (medium read lengths, medium throughput, and medium cost), as well as systems by Solexa, Helicos, and Applied Biosystems (ABI) (high throughput, low cost, short reads), compare with one another. Some applications, such as *de novo* sequencing are currently amenable only to systems with longer read lengths, while other applications (gene expression and resequencing) are well served by large volumes of 25–35 base reads.

sequence of the template DNA, a process also known as sequencing by synthesis. The template for pyrophosphate-based sequencing, or pyrosequencing, reactions is a homogenous population of DNA molecules to which oligonucleotide primers have been annealed. DNA polymerases are added to the reaction and they bind to the hybridized primers after which each of the four nucleotide triphosphates is sequentially added to the sequencing reaction. The sequential addition of the four individual nucleotides is repeated cyclically for the length of the sequencing run. When any given nucleotide triphosphate added to the reaction is complementary to the base at the position directly downstream of the primer, the polymerase will incorporate it into the growing priming strand, releasing pyrophosphate in the process. If the stretch of bases directly downstream from the primer is composed of only a single base complementary to the added nucleotide, only a single base is incorporated, a single pyrophosphate is released, the polymerase advances, and the length of the priming strand increases by a single base. However, if the template downstream of the primer is a homopolymeric region, composed of multiple contiguous bases complementary to the added nucleotide, the polymerase will advance and extend the primer for as many bases as comprise the homopolymer, and inorganic pyrophosphate (PPi) molecules will be released proportionate to the number of bases in the homopolymer. If the added nucleotide is not complementary to the base 3′ of the primer, no nucleotide is incorporated, no PPi is released, and the polymerase pauses until a complementary nucleotide is added.

The nucleotide incorporation-dependent PPi release initiates an enzyme cascade illustrated by the equation below, wherein the PPi and adenosine phosphosulfate (APS) present in the reaction mix are converted to ATP by ATP sulfurylase (see equations below, where *n* indicates the number of nucleotides). The ATP, along with oxygen and D-luciferin (also present in the reagent stream), is subsequently converted to oxyluciferin and photons through the activity of luciferase. The number of photons generated by the cascade within any PicoTiterPlate well is directly proportional to the PPi concentration and also to the number of nucleotides incorporated by the polymerase.

$$(\mathrm{DNA})_n + \mathrm{dNTP} \xrightarrow{\text{DNA polymerase}} (\mathrm{DNA})_{n+1} + \mathrm{PPi}$$

$$\mathrm{PPi} + \mathrm{APS} \xrightarrow{\text{ATP sulfurylase}} \mathrm{ATP} + \mathrm{SO_4}^{2-}$$

$$\mathrm{ATP} + \text{luciferin} + \mathrm{O_2} \xrightarrow{\text{Luciferase}} \mathrm{AMP} + \mathrm{PPi} + \text{oxyluciferin} + \mathrm{CO_2} + h\nu$$

The 454 sequencing is performed within a PicoTiterPlate, made of approximately 1.6 million individual optical fibers fused into a single piece of glass. A partial etching process converts each of the optical fibers into a 55 μm-deep, 75 pl reaction well on one end, while leaving the other side of the PicoTiterPlate optically smooth. The 454 sequencing reactions occur simultaneously under a cyclic flow of reagents with the face of the PicoTiterPlate wells open to the reagent flow and the unetched side of the plate in contact with a charge coupled device (CCD) camera. The use of optical fibers for the reaction well provides a direct connection between the reaction wells in the PicoTiterPlate, and the CCD camera permits precise quantification of the amount of light generated within each well. The amount of light generated during each nucleotide flow at each well on the

PicoTiterPlate is measured for the duration of the run. These data are then subjected to software normalization, filtering, correction, and deconvolution to reveal the template sequenced within each of the light-generating wells.

Preparation of large templates for the 454 system incorporates some streamlined aspects of whole-genome shotgun sequencing in that the template is randomly fragmented, but completely avoids any cloning steps, permitting process completion in 24 h or less. Template DNA is mechanically fractionated through nebulization and size-selected to produce a population of double-stranded fragments ranging from 400 to 600 bases. Two distinct types of oligonucleotide adapters are then ligated onto the fragments, one of which is biotinylated, providing priming sites for subsequent amplification and sequencing as well as permitting collection of single-stranded templates possessing heterogeneous adapters on the ends of the fragment. Preparing PCR products for sequencing is a simpler process, involving bipartite primers with product-specific $3'$ portions and a 454-specific $5'$ tag.

Recently 454 has also developed a paired-end sequencing protocol enabling the determination of the relative positions and orientation of contigs during *de novo* sequencing. In summary, the process involves fragmentation of the template genome to produce 2.5-kb-long templates, to either end of which adaptors are ligated. The fragments are then circularized and cleaned with Mme1 restriction sites present in the adaptors, producing 84-base-pair-long fragments comprising an adaptor sequence separating two 20 base sequences originally located on either end of the 2.5 kb fragment.

Regardless of the method of preparation, the templates are clonally amplified and immobilized on 28 μm DNA capture beads through emulsion PCR or emPCR. The emPCR process utilizes a limiting dilution of template that is applied to a solution of PCR reaction mix and DNA capture beads, emulsified in a thermostable oil matrix, and thermocycled. The limiting dilution ensures that the majority of the droplet-based microreactors contain no template molecules, but those that do contain a template molecule contain only one. Amplification of each template molecule in a discrete microreactor permits unbiased amplification of complex samples, as templates are amplified individually rather than in bulk. As a result, differential amplification efficiencies, influenced by product length, GC content, and so on, are minimized. Only droplets that contain a DNA capture bead and a viable template generate amplicons that are immobilized on the DNA capture bead and are subsequently enriched away from the beads lacking the amplified template. The enriched beads are then loaded into the wells of the PicoTiterPlate for sequencing.

One of the main benefits of the 454 sequencing system is its rapidity and cost efficiency compared to the existing capillary array systems. As at the time of this writing, a single FLX produces 200 Mb of data per day at a cost of roughly $160 per megabase, compared to approximately 1.4 Mb per day at around $900 per megabase with standard capillary array units. Sequences generated by the 454 system have also been shown to be remarkably unbiased compared to those generated using previous technologies. As previously mentioned, 454 sequencing avoids cloning and amplification-induced bias through the emPCR process. Bias inherent to the sequencing process itself, such as poor coverage of GC-rich areas and hard stops in regions with high levels of secondary structure, is reduced through the use of the strand-displacing *Bst* polymerase during 454 sequencing, as opposed to the use of less tenacious polymerases in traditional Sanger sequencing. The types of errors generated in 454 sequencing are different from those encountered during traditional sequencing; because the individual nucleotides are flown cyclically, the possibility for polymerases to misincorporate nucleotides is substantially decreased. As a result, substitution type errors are rarely encountered in 454 sequencing; if an error does occur, it will most likely be an insertion/deletion (or indel) type error, as had occurred with Sanger's original plus–minus sequencing technique. Finally, pyrophosphate-based sequencing is immune to the electrophoresis-specific biases such as GC compression that affect both gel- and capillary-based systems.

Limitations of the 454 sequencing system must be addressed as well, some of which are inherent in the method while others are symptomatic of any developing technology and may be corrected over time. When compared to the 500–800 base reads produced by conventional sequencing, the 454 system's most apparent limitation is the short length of the sequences (100–125 for the GS-20, 200–300 for the FLX) that they generate. The shorter reads present challenges for assembling *de novo* sequences primarily because they have more difficulty spanning repeat sections than longer reads and are less likely to contain sequences that can be unambiguously placed within an assembled scaffold or contig. This limitation has been partially addressed by the development of the paired-end library for 454 sequencing, which facilitates contig orientation within scaffolds by generating sequence pairs separated by known lengths of intervening sequences, but it does nothing to improve the placement of individual reads. In a more mundane fashion, shorter reads are more difficult to manage while sequencing as it requires more of them to completely cover large genomes, and present more potential permutations that assembly software must consider, requiring additional CPU time and capacity.

Another limitation of the 454 system involves sequencing homopolymer regions, in terms of both physical parameters and accurate data analysis. When a homopolymer is encountered during pyrophosphate-based sequencing, the polymerase will extend along the entire length of the stretch, releasing PPi proportional to the number of bases in the homopolymer. The relationship between homopolymer size and the number of photons generated by the sulfurylase–luciferase enzyme cascade is linear for homopolymers up to eight nucleotides, after which the relationship for individual reads begins to decay. This stems from the fact that basecalls for the 454 system are derived from the intensity of the emitted signal from a given well during a particular nucleotide flow. While the accuracy of that basecall is dependent on signal intensity, the variability of signal intensity is also proportional to the signal strength. As homopolymer length and signal intensity increase, the signal variability grows as well, increasing the probability of error when using the signal intensity to call the actual number of bases in the homopolymer. Typical 454 sequencing runs can identify a single base with greater than 99.9% accuracy; 2-base homopolymers are correctly identified 99.5% of the time and 3-base homopolymers 99.0% of the time. For 9-base homopolymers, the accuracy of basecalling has degenerated such that the basecalls are roughly 64% accurate. It is important to note, however, that this pertains to single reads where only the accuracy of the read from a single well is considered, and that 99% of these 9-mers are called as 8-, 9-, or 10-base homopolymers. The random nature of the signal variability, where erroneous basecalls are as likely to be too high as too low, allows the use of oversampling to correct homopolymer error. When multiple reads covering the same homopolymer are averaged, the consensus basecall accuracy for that 9-base homopolymer is close to 99%.

The high throughput provided by both the GS-20 and the FLX have enabled projects that were considered impractical using conventional sequencing. Since its commercial release in 1995, 454 sequencing has been used for more than 60 peer-reviewed papers, including metagenomic investigations of deep-mine ecology, the deep sea, and solar salterns, and the identification of the virus responsible for colony collapse disorder by a metagenomic sequencing of the bees themselves. The system has been employed for standard genomics including *de novo* sequencing of multiple microbes (e.g., *C. jejuni, Helicobacter pylori*, and bacteria from the Antarctic Sea), and has also been successfully utilized for studies of gene expression and DNA methylation in multiple species of organisms.

Solexa

The second of the next-generation sequencers to be commercially released was the 1G released by Solexa in early 2007, generating up to 1 Gb of data through 40 million 25 base reads in a 72-h run. Like the GS-20 and the FLX, the 1G is a sequencing-by-synthesis system, but unlike the 454 systems it relies on incorporation of fluorescent, reversible chain-terminating nucleotides for signal generation.

As with 454, the sample preparation process involves random fragmentation of the template, with subsequent ligation of adaptors that serve as forward and reverse primers for PCR and sequencing at either end. Single-stranded template molecules are covalently attached at a density of approximately 100 million templates per square centimeter to the surface of a glass slide to which lawns of forward and reverse primers have also been bound. The templates are then amplified to roughly 1000 copies through solid-phase amplification, also known as bridge amplification, a version of PCR that utilizes the immobilized rather than the solution-phase PCR. Through bridge amplification, the free end of a given immobilized template hybridizes to a proximal immobilized primer. As in traditional PCR, a polymerase generates a nascent copy of the template molecule, bound to the glass surface by the 5′ end of the elongating primer. Each denaturation phase in the PCR cycle disassociates the double-stranded products, which are then free to hybridize to other complementary immobilized primers. If bridge amplification reactions are run to completion, the templates will amplify from every immobilized primer within the reach of the molecule, at a distance that must be shorter than the length of the template itself. During amplification, products expand outward from the original immobilized template across the surface of the slide, generating circular colonies of amplified template.

After amplification is complete, sequencing is accomplished through the simultaneous addition of all four chain-terminated nucleotides, each possessing a distinct fluorophore, which are incorporated into the complementary strand by polymerases engineered to accept modified nucleotides. After the incorporation phase, the entire slide is scanned with a laser and the fluorescence is measured for each colony of templates, the fluorescent terminating moiety is chemically removed, and the process is repeated for each subsequent position on the template. The use of chain-terminated nucleotides prevents the homopolymer errors that affect 454 sequencing, in that each base of a sequence is read individually whether part of a homopolymer stretch or not; however, this allows substitution as well as insertion/deletion errors to occur during sequencing.

The overriding benefits provided by the Solexa's 1G sequencing system are throughput and cost. With the capability of generating up to 1 Gb of data per run at a cost of approximately $5 per megabase, Solexa delivers significantly more data at a substantially lower price per base than any of the existing Next-Gen or conventional sequencing technologies. Sequencing output of this magnitude has obvious applications where large numbers of reads are desired for achieving sufficient coverage depth when resequencing and accurately quantifying transcripts within complex populations for expression-profiling projects. In each of these contexts, the 25–35 base reads generated by the Solexa system are sufficient. For resequencing, the reads are simply mapped against a known target sequence, and any discrepancies are identified. The 25 base reads are adequate to identify most of the expressed genes.

Due to the scarcity of published data for the Solexa system, the degree to which the system will be able to sequence hard motifs is currently unknown, and is likely dependent on strand-displacing activity of the polymerase and the temperature at which sequencing is conducted.

Despite the use of engineered polymerases, the modified nucleotides are still not incorporated as readily as natural nucleotides and this, in conjunction with the cumulative loss of signal due to incomplete cleavage of the terminators and fluorophores from the incorporated nucleotide, limits read lengths to between 25 and 35 nucleotides, roughly 10% of that possible with the 454 FLX sequencer.

As mentioned previously, reads of this length are suitable for resequencing application, but are of limited value when attempting *de novo* sequencing owing to the difficulty in obtaining overlapping sequences of sufficient length to tile reads when forming contigs, and their inability to span nucleotide repeat sections longer than 35 bases.

The run durations (72 h) are considerably longer for the Solexa instrument than for capillary array sequencers or the 454 system. Although the 1G generates an enormous amount of data for the specific samples sequenced, the run duration limits the number of samples that can be analyzed per day.

Perversely, the sheer volume of data generated by the 1G might limit its accessibility to ordinary laboratories. Generating terabyte amounts of data per run, the 1G requires an entire network of computers for analysis and data storage, infrastructure more commonly found in genome centers and core lab facilities.

Applied Biosystems

Applied Biosystems (ABI) entered the next-generation sequencing business with the acquisition of Agencourt in 2006 and launched an early-adopter program for the SOLiD technology in 2007. No published data exist on the system as yet, but from what can be obtained from the company website, the SOLiD system can generate up to 3 Gb of data during a 6-day run consisting of fewer than 100 million individual 35 base pair reads. SOLiD technology utilizes a significantly different method for sequencing, employing a ligase-driven ‘sequencing-by-ligation’ technique as opposed to the polymerase-mediated ‘sequencing-by-synthesis’.

The sample preparation and amplification processes are very similar to those used in 454 sequencing, with template DNA fragmented into one of two size ranges depending on whether simple fragment or paired-end libraries are required; fragment libraries are roughly 70 bases in length, while paired-end libraries are 1–6 kb. As in the emPCR process, individual templates of a limiting dilution are clonally amplified and immobilized on DNA capture beads through emulsion PCR, although the beads are polystyrene rather than Sepharose and smaller, measuring 1 μm in diameter. Following emPCR, the small percentage of DNA capture beads possessing immobilized PCR amplicons are enriched from the total bead population and anchored with biotin–streptavidin linkages to the surface of a glass slide. The slide is then loaded into a flowcell on the SOLiD instrument for subsequent sequencing.

The sequencing process requires addition of primers specific to the 5′ end of the amplified templates and a mix of four different 8-base oligonucleotide probes, where each of the four probe types carries a distinct 3′ fluorophore. After the priming sequencing has annealed to the priming site, the oligo probes anneal to the template directly downstream from the sequencing primer. Probe hybridization is specific only for the two bases in the 4th and 5th positions on the probe. Probes that contain 4th- and 5th-position bases complementary to the two corresponding bases on the template anneal to the template and are ligated onto the sequencing primer, while noncomplementary probes are washed away. The fluorescent signal generated by the hybridized probe is then detected and recorded. The fluorescent signal detected through this process is reflective of the sequence at a specific location on the sequencing template.

The ligated probe is then cleaved between the 5th and 6th bases, removing the fluorescent moiety, and a second round of probe ligation occurs. In this round, the probes’ 4th and 5th base specificity interrogates the nucleotides 9 and 10 bases downstream from the end of the sequencing primer, and the probe is ligated onto the tail of the cleaved probe from the previous round of sequencing.

This process is repeated several times, after which the sequencing primer and the associated train of ligated probes are stripped off the template, and the process is repeated with a sequencing primer one base shorter than the previous sequencing primer. By reducing the length of the sequencing primer, template bases 3 and 4 are sequenced in the first round, 8 and 9 in the second, and so on. In this fashion a total of five sequencing primers of different lengths are employed, generating up to 35 base reads.

According to company literature, the system is expected to produce sequences that are greater than 99.94% accurate when using 2-base encoding, and a consensus accuracy of 99.999% when the sequence coverage exceeds 15× oversampling. Interestingly, the company also reports that of the reads generated during a run, 50% or more will have 0 or 1 error when aligned to a reference sequence, and that 50% or less of the beads will have 2 or 3 errors when aligned to reference sequences. Using the maximum 35 base read length, this implies that 50% or more of the reads will have an accuracy of 97% or greater, while 50% or less of the reads will possess accuracies as low as 94% and 91%.

Until the SOLiD system is released for widespread use and generates peer-reviewed publications, it is difficult to accurately assess the system's benefits and limitations. One can suppose that the SOLiD will generate data on a gigabase scale composed of large numbers of short reads, an inference supported by the fact that the system ships with its own 10-unit Linux cluster to enable data analysis. The ABI system is expected to provide benefits similar to that obtained with the Solexa 1G: massive amounts of sequence data generated at extremely low costs per base. Also like the 1G, the short read lengths will probably preclude the system's use for *de novo* sequencing application. It is possible that the short read lengths result from the gradual accumulation of incompletely cleaved probes, and that improvements to this process may increase read lengths.

Helicos

Another Next-Gen sequencing system approaching commercial release is Helicos BioSciences' HeliScope; as with the ABI SOLiD, there is little published information on the system at this time, aside from what can be obtained from the company website. Based on zero wave guide technology developed by Steven Quake, the HeliScope differs from the previously described sequencing processes, both traditional and next-generation, in that the template DNA is not amplified prior to sequencing.

Like the previous systems, sample preparation involves fragmentation of the target genome into sections several hundred bases in length, and either adapters are ligated onto the ends of each fragment or a poly(A) tail is added, depending upon the sequencing application. Approximately 1×10^9 single-stranded templates are then annealed to probes that are already chemically anchored to the surface of a glass slide. These probes are either a reverse complement oligo of the ligated adaptor (for templates to which adaptor sequences were ligated) or a poly(T) oligo (for templates to which poly(A) tails were attached). For each nucleotide cycle, polymerases are bound to the immobilized templates, and both fluorescently labeled nucleotides and proprietary terminators that kinetically inhibit the polymerase from incorporating more than a single base are added. After single fluorescent nucleotides are incorporated, the slide surface is imaged with a high-intensity laser where the fluorescent signal detected indicates a base incorporation at that position in the fluorescing template sequence.

As only a single template molecule is interrogated per sequence, rather than the clusters of thousands to millions of clonally amplified molecules analyzed per template with the other sequencing systems, extremely sensitive detection methods must be employed. Helicos uses total internal reflection microscopy (TIRM), which constrains the physical space interrogated to a region within 150 nm of the flowcell surface. This substantially reduces the background fluorescence that is typically generated by bulk solutions and, in conjunction with substantial washing to remove errant signals from unincorporated nucleotides, enables accurate detection of single fluorescent molecules.

While sequencing single-molecule, templates encounter technical challenges not faced by clonal population sequencing; distinct benefits are also accrued. For example, when sequencing clonal populations, not all templates in the population advance at the same rate, and a general loss of phase is inevitable during a sequencing run. This loss of phase, when some percentage of the total polymerase molecules might be located at position n on the template, another at position $n-1$, and a third at position $n+1$, causes loss of signal and an increase in background, eventually limiting read lengths. When sequencing single strands, loss of phase is not possible, although polymerase-related problems such as insertions, where an incorrect base is inserted along with the proper base (these should be relatively rare in the Helicos system), deletions, where either a base is not incorporated when it should have been or the proper nucleotide is incorporated but lacks the fluorescent label and so is not recorded, and misincorporations/substitutions, where an incorrect base is incorporated, can occur and result in either erroneous sequence or premature termination of that template's read. No throughput specifications for the HeliScope have been released yet, but with approximately 1×10^9 anchored templates, reads on par with Solexa and SOLiD could theoretically generate 25 Gb per run.

Combined Approaches

Each of the technologies described, whether traditional or next-generation, possesses a combination of factors such as read length, throughput, and cost that make them suitable for particular applications in genomic research, as illustrated in **Figure 4**. For example, the long read lengths delivered by traditional capillary sequencing are well suited for *de novo* sequencing projects, but the cost per base and the extensive infrastructure required to obtain high-throughput levels with the technology makes it less amenable to gene expression or resequencing applications where large numbers of reads are required. Solexa or ABI reads, on the other hand, are too short for *de novo* sequencing, but the systems' exceptional throughput and affordable cost per base make them an obvious choice for resequencing and expression profiling. The 454 system occupies a middle ground, supporting multiple applications; although its reads are shorter than the capillary system's, and its cost per base higher than Solexa's and ABI's, it is a viable platform for applications ranging from gene expression to *de novo* sequencing.

While each of the systems has a particular mix of strengths and weaknesses, discussion of applications enabled by any single system may be somewhat artificial as maximum efficiency and accuracy will likely be obtained by using a combination of technologies. For example, use of traditional Sanger sequencing along with Next-Gen data may provide the best alternative for maximizing speed, cost, and completeness of coverage when sequencing whole genomes. Researchers have already demonstrated that a hybrid approach using a mixed next-generation (in this case, reads from the GS-20) and traditional sequencing provided the optimum combination of cost, completeness of coverage (due to lack of cloning bias), and total assembly when sequencing multiple species of microbes. It is highly likely that similar benefits would result from a combination of Sanger sequencing with any of the high-throughput systems.

Future Directions

The genomics field is in the midst of a period characterized by the rapid emergence of multiple sequencing platforms and techniques. In addition to the improved accuracy, throughput, and cost effectiveness provided by continuous refinement of existing systems, promising novel technologies such as nanopore sequencing are in active development. Introduction and incorporation of any disruptive technology is accompanied by some amount of inconvenience and effort, as users are forced to educate themselves about the advantages and costs associated with the new techniques. In addition, the genomics community has had to not only evaluate the flood of data generated by the different platforms, but also develop methods to efficiently incorporate disparate data types into completed genomes and universal databases. Despite the inconveniences inherent and the effort required in adopting new technologies, the benefits are evident not only in the rising number of microbes either completely sequenced or subjected to routine resequencing and the dramatic reduction in sequencing costs, but also in the development of entirely new applications that were simply impossible with the previous technologies. Advances in microbial genomics can have a profound impact on human health. If the rate of technological innovations is proportional to the utility and importance of their benefits, then the next 10 years should be exciting indeed.

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Emerging Infections

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A 30-Year Perspective
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Further Reading

Glossary

amplification of transmission The increased spread of infectious diseases that occurs naturally or because of facilitating factors, such as nonsterilized needles and syringes, that can result in an increase in transmission of infections such as hepatitis.

anti-infective drug resistance The ability of a virus, bacterium, or parasite to defend itself against a drug that was previously effective. Drug resistance is occurring in bacterial infections such as 'tuberculosis' and 'gonorrhoea', in parasitic infections such as 'malaria', and in viral infections such as AIDS.

emerging infection A newly identified and previously unknown infectious disease in humans, often resulting from a breach in the species barrier between humans and animals that carry the infectious agent. Since 1970, there have been over 40 emerging infections identified, causing diseases ranging from diarrheal disease among children, hepatitis, and AIDS to Ebola and Marburg hemorrhagic fevers.

eradication The complete interruption of transmission of an infectious disease and the disappearance of the virus, bacterium, or parasite that caused the infection. The only infectious disease that has been eradicated is 'smallpox', which was certified as eradicated in 1980.

International Health Regulations (2005) International law that is intended to protect against the spread of infectious diseases across international borders, aimed at ensuring global public health security. The regulations provide new standards and norms for national and global disease surveillance, notification, and response; and require reporting to the World Health Organization (WHO) of all public health emergencies of international concern, including events or hazards arising from communicable diseases, biological, radionuclear, and chemical agents.

reemerging infection A known infectious disease that had fallen to such low prevalence or incidence that it was no longer considered a public health problem, but that is presently increasing in prevalence or incidence. Reemerging infections include 'tuberculosis', which has increased worldwide since the early 1980s, dengue in tropical regions, and 'diphtheria' in Eastern Europe.

Abbreviations

BSE	bovine spongiform encephalopathy
IHR	International Health Regulations
SARS	severe acute respiratory syndrome
WHO	World Health Organization

A 30-Year Perspective

Infectious diseases, whether caused by bacteria, viruses, or parasites, are complex, dynamic, and constantly evolving. Some emerge or reemerge in human populations as they cross the species barrier from animals to humans, and once they have infected humans they may be asymptomatic or cause disease. If they cause disease, they may maintain their virulence or decrease in virulence with further passage through human populations. While some of these infectious agents transmit easily from human to human causing epidemics or pandemics, others may not be transmissible, but continue to sporadically infect humans as zoonotic infections. Still others, like HIV infection, may eventually become endemic infectious diseases in humans.

Examples of emerging infectious diseases are numerous and clearly demonstrate their complexity, dynamism, and evolution. In the Democratic Republic of Congo, the cessation of smallpox vaccination in 1980, after smallpox had been eradicated, may have contributed to a change in the transmission patterns of human monkeypox. Smallpox vaccine also protects against other orthopox virus infections of humans, including monkeypox. During the 1970s and 1980s, when human monkeypox was the subject of extensive study and the majority of humans were still vaccinated against smallpox, it was shown that person-to-person transmission beyond three generations was rare. In 1996–97, an outbreak of human monkeypox occurred with transmission through at least nine generations and probably more, and large chains of human to human transmission of monkeypox have continued intermittently since then. Poverty and civil unrest appear to be factors that contribute to the increase in human monkeypox infections, and these infections are now better able to sustain transmission because succeeding generations are no longer vaccinated.

Numerous other infectious diseases have likewise shown the potential to emerge, reemerge and transmit from human to human (**Table 1**). In the early to mid-1970s, for example, classic dengue fever had just begun to reappear in Latin America after it had been almost eliminated as a result of mosquito control efforts in the 1950s and 1960s. During the early 1970s, dengue continued to reemerge, with unprecedented numbers of its hemorrhagic form. Thirty years later, in 2001, Latin America reported over 609 000 cases of dengue, of which 15 000 were of the hemorrhagic form. These figures represent more than double the cases reported for the same region in 1995.

Dengue outbreaks are also becoming more numerous and lethal. An outbreak in Brazil in 2002 caused over 500 000 cases in one of the largest outbreaks ever recorded. In 1998, over 1.2 million cases of dengue fever and dengue hemorrhagic fever were reported from 56 countries. Today, dengue is occurring in epidemics in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Dengue outbreaks in Indonesia began during the early 2000s, and have resulted in over 60 000 reported cases with more than 700 deaths in this country alone.

In 1991, cholera, which had not been reported in Latin America for over 100 years, reemerged in Peru with over 320 000 cases and nearly 3000 deaths. It rapidly spread throughout the continent to cause well over 1 million cases in a continuing and widespread epidemic. Fifteen years earlier, in 1976 in North America, *Legionella* infection was first identified in an outbreak among war veterans attending a conference in Philadelphia (USA). *Legionellosis* is now known to have occurred in outbreaks many years prior to that date. Today it occurs worldwide, posing a threat to travelers exposed to water from many different sources including poorly maintained air conditioning systems because the organisms are resistant to chlorination. In the Netherlands, in 1999, an outbreak of *Legionellosis* occurred, which was subsequently traced to exposure to mist from whirlpool baths exhibited at a flower show visited by 80 000 people. Local cooling towers are considered the likely source of a large outbreak of *Legionellosis*, involving 751 cases and two deaths, which occurred in Spain in 2001.

In 1986 a new disease in cattle, bovine spongiform encephalopathy (BSE), was first identified in the United Kingdom. In 1996, the appearance of a previously unknown variant of the invariably fatal Creutzfeldt-Jakob disease appeared in humans, and this has now been shown to be caused by the same infectious agent that causes BSE in cattle. Some scientists have suggested that BSE may have been the result of cross species transmission from sheep as the result of feeding cattle animal proteins from dead sheep. By mid-2006 variant Creutzfeldt-Jakab disease had occurred in over 195 persons, and infected cattle had been identified in 24 additional countries.

Within a decade, food-borne infection by *Escherichia coli* 0157, unknown in the 1970s, had become a food-safety concern in Japan, Europe, and in the Americas. Hepatitis C was first identified in 1989 and is now thought to be present in at least 3% of the world's population, while hepatitis B has reached levels exceeding 90% in populations at high risk from the tropics to Eastern Europe.

In 2003, the severe acute respiratory syndrome (SARS) coronavirus was first identified, demonstrating the full potential of emerging infectious agents for international spread. Thought to be an animal virus from a yet unproven animal reservoir, the SARS coronavirus first infected humans in the Guangdong province of China in 2002. From China it rapidly spread to 26 countries, and resulted in over 8000 human cases during 2003.

In the Hong Kong Special Administrative Region of China, 18 cases of human zoonotic infection with influenza A virus subtype H5Nl occurred in 1997. Six of these cases were fatal. The H5N1 virus had been previously confined to wild bird populations, but around 1997 it began to infect domestic poultry in China and Hong Kong. By 1 December 2007, poultry infections were occurring throughout Asia, the Middle East, Europe, and Africa, and a total of 335 human zoonotic infections of H5N1 had occurred, of which 206 were fatal. The threat of emergence of a global influenza pandemic in humans continues, either as a result of adaptive mutation or reassortment of the H5N1 virus as it continues to circulate in nonhuman mammals and avian populations, or from one of the other avian influenza viruses now circulating in avian populations.

Human African trypanosomiasis, which had been virtually eliminated in the 1960s, resurged in an epidemic that in 1998 was thought to have infected 300 000 to 500 000 people. In 1976, the Ebola virus was first identified in simultaneous outbreaks of hemorrhagic fever in Democratic Republic of Congo and Sudan. In many ways, Ebola hemorrhagic fever has come to symbolize emerging diseases and their potential impact on human populations

Table 1 Principal newly identified infectious organisms associated with diseases

Year	*Newly identified organism*	*Disease (year and place of first recognized or documented case)*
Diseases primarily transmitted by food and drinking water		
1973	Rotavirus	Infantile diarrhea
1974	Parvovirus B19	Fifth disease
1976	*Cryptosporidium parvum*	Acute enterocolitis
1977	*Campylobacter jejuni*	Campylobacter enteritis
1982	*Escherichia coli* 0157:H7	Hemorrhagic colitis with hemolytic uremic syndrome
1983	*Helicobacter pylori*	Gastric ulcers
1986	*Cyclospora cayatanensis*	Persistent diarrhea
1989	Hepatitis E virus	Enterically transmitted non-A and non-B hepatitis (1979, India)
1992	*Vibrio cholerae* 0139	New strain of epidemic cholera (1992, India)
Unclear modes of transmission, thought to be primarily transmitted by drinking water		
1985	*Enterocytozoon bieneusi*	Diarrhea
1991	*Encephalitozoon hellem*	Systemic disease with conjunctivitis, in AIDS patients
1993	*Encephalitozoon cunicali*	Parasitic disseminated disease, seizures (1959, Japan)
1993	*Septata intestinalis*	Persistent diarrhea in AIDS patients
Diseases primarily transmitted by close contact with infectious individuals, excluding sexually transmitted diseases, nosocomial infections, and viral hemorrhagic fevers		
1980	HTLV-1	T-cell lymphoma-leukemia
1982	HTLV II	Hairy cell leukemia
1988	HHV-6	Rosela subitum
1993	Influenza A/Beijing/32 virus	Influenza
1995	HHV-8	Associated with Kaposi sarcoma in AIDS patients
1995	Influenza A/Wuhan/359/95 virus	Influenza
2003	SARS coronavirus	SARS
Sexually transmitted diseases		
1983	HIV-1	AIDS (1981)
1986	HIV-2	Less pathogenic than HIV-1 infection
Nosocomial and related infections		
1981	Staphylococcus toxin	Toxic shock syndrome
1988	Hepatitis C	Parenterally transmitted non-A, non-B hepatitis
1995	Hepatitis G viruses	Parenterally transmitted non-A, non-B hepatitis
Human zoonoses and vector-borne diseases, including viral hemomorrhagic fevers, transmitted by close contact with animals or animal products, excluding food-borne diseases		
1977	Hantaan virus	Hemorrhagic fever with renal syndrome (1951)
1990	Reston strain of Ebola virus	Human infection documented but without symptoms (1990)
1991	Guanarito virus	Venezuelan hemorrhagic fever (1989)
1992	*Bartonella henselae*	Cat-scratch disease (1950s)
1993	Sinnombre virus	Hantavirus pulmonary syndrome (1993)
1994	Sabiä virus	Brazilian hemorrhagic fever (1955)
1997	Influenza A (H5N1)	Avian influenza
1998	Nipah virus	Severe encephalitis
Tick-borne		
1982	*Borrelia burgdorferi*	Lyme disease (1975)
1989	*Ehrlichia chaffeensis*	Human ehrlichiosis
1991	New species of *Babesia*	Atypical babesiosis
Unknown animal vector		
1977	Ebola virus	Ebola hemorrhagic fever (1976, Democratic Republic of Congo and Sudan)
1994	Ebola virus, Ivory Coast strain	Ebola hemorrhagic fever
Soil-borne diseases, airborne diseases and diseases associated with recreational water with no evidence of direct person-to-person transmission		
1976	*Legionella pneumophila*	Legionellosis (1947)

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without previous immunological experience. The largest recorded outbreak, which began in Uganda in 2001, caused 425 confirmed cases and 224 deaths. Altogether, since 1976 Ebola has caused just under 2000 human infections of which approximately 1300 have been fatal, and epidemics continue to occur in west, central, and east African countries.

A retrospective analysis in 1985, of blood that had been collected from persons living in communities around the site of the 1976 Ebola outbreak, demonstrated that HIV seroprevalence was already almost 1%. HIV has since become a preoccupying problem in public health worldwide. Widespread infection with HIV, an infectious agent that is now endemic and appears to have emerged in human population sometime during the first half of the twentieth century, provides fertile ground for the transmission of tuberculosis, including extensively drug resistant-tuberculosis (XDR-TB), because HIV kills CD4 lymphocytes and destroys much of the acquired immune system. Drug-resistant tuberculosis has been identified in all regions of the world but is today most frequent in the countries of the former Soviet Union and in Asia, and is on the rise in Africa.

Misplaced Optimism

With the certification of the eradication of smallpox in 1980, an unparalleled public health accomplishment that resulted in immeasurable prevention of human suffering and death, there was great optimism that infectious diseases were no longer a major threat. Vaccines and antimicrobial drugs that became available as the result of intensified research and development immediately following World War II provided further optimism, as well as technological advances in hygiene and sanitation.

Malaria, endemic in many industrialized countries in both North America and Europe, disappeared with the use of insecticides and antimalarial drugs. Tuberculosis hospitals in Europe and North America emptied as living conditions improved and effective drugs became available that could be used to treat those with acute tuberculosis and prevent infection in their contacts. Influenza deaths could be prevented in industrialized countries by vaccinating elderly populations, and epidemics of diarrhoeal disease became rare events, limited to foodborne outbreaks when temporary breaches in sanitation occurred.

This optimism resulted in a transfer of resources and infectious disease specialists away from infectious disease control and public health. More and more resources were invested in the development of drugs useful for noncommunicable diseases related to lifestyle and aging, and public health infrastructure weakened. At the same time, however, with increasing use of antimicrobial drugs, warning signs of microbial resilience began to appear and increase in magnitude.

At the end of the 1940s, resistance of hospital strains of *Staphylococcus* to penicillin in the United Kingdom had become as high as 14%. By the end of the 1990s it had risen to levels of 95% or greater, and by 2000 multidrug-resistant staphylococcal infections had become the cause of great public health concern. In New York City in the 1990s, multidrug-resistant strains of tuberculosis gained their hold in hospitals, prisons, and homeless populations. At the same time, multidrug-resistant tuberculosis emerged in the Russian Federation and the prevalence more than doubled in less than seven years, with over 20% of tuberculosis patients in prison settings infected with multidrug-resistant strains. Development of extensively drug-resistant tuberculosis rapidly followed, and by 2006 had become a major public health problem in southern Africa.

Levels of anti-infective drug resistance of *Staphylococcus aureus* and other microbes increased with great rapidity. By 1976, chloroquine-resistant *Plasmodium falciparum* malaria was highly prevalent in southeastern Asia and 20 years later was found worldwide, as was high-level resistance to two back-up drugs, sulfadoxine-pyrimethamine and mefloquine. Antimicrobial drugs developed to treat AIDS and other sexually transmitted infections such as gonorrhoea likewise began to lose their efficacy because of the rapid development of resistance. In the early 1970s, *Neisseria gonorrhoeae* that was resistant to usual doses of penicillin was just being introduced into Europe and the United States from Southeast Asia, where it is thought to have first emerged. By 1996, *N. gonorrhoeae* resistance to penicillin had become worldwide, and strains resistant to all major families of antibiotics had been identified wherever these antibiotics had been widely used. Countries in the Western Pacific, for example, have registered quinolone resistance levels up to 69%.

As a result of the shift in resources to other health priorities, vaccine and antimicrobial drug development lagged. No effective vaccines have been developed to prevent infection of many of the major mortality causing infections such as tuberculosis, malaria, and AIDS, and research and development of new antimicrobial agents has slowed.

Optimism is now being replaced by an understanding that the world is less well equipped to deal with infectious diseases because of emerging anti-infective drug resistance, and because the infrastructure for infectious disease surveillance and control has suffered and in some cases become ineffective. These weaknesses have recently come into sharp focus as countries consider preparedness plans for responding to the possible deliberate use of biological agents or a potential influenza pandemic, and recognize the importance of strong public health systems as the first line of defense for infectious disease outbreaks, irrespective of their origin.

Public Health Weakness Facilitating Emergence and Reemergence

The weakening of the public health infrastructure for infectious disease control is evidenced by the failures in mosquito control in Latin America and Asia that facilitated the reemergence of dengue that is now causing major epidemics. It was also evidenced in disinvestment in childhood immunization programes in Eastern Europe during the 1990s, which contributed to the reemergence of epidemic diphtheria; and in Africa and Latin America where yellow fever vaccination coverage markedly decreased, facilitating yellow fever outbreaks on both continents, the most severe of which was a major urban outbreak in Cote d'Ivoire in 2001.

Weakening of public health infrastructure is also clearly demonstrated by the high levels of hepatitis B and the nosocomial transmission of other pathogens such as HIV in the former Soviet Union and Romania, and the nosocomial amplification of outbreaks of Ebola in Democratic Republic of Congo, where syringes and failed barrier nursing amplified outbreaks into major epidemics.

Population increases and rapid urbanization have likewise resulted in the breakdown of sanitation and water systems in large coastal cities in Latin America, Asia, and Africa that promoted the transmission of cholera and shigellosis. In 1950, there were only two urban areas in the world with populations greater than 7 million, but by 2005 this number had risen to 34, with increasing populations in and around all major cities, challenging the capacity of existing water and sanitary systems.

Anthropogenic or natural effects on the environment also contribute to the emergence and reemergence of infectious diseases. The effects range from global warming and the consequent extension of vector-borne diseases, to ecological changes due to deforestation that increase contact between humans and animals and the possibility of microorganisms breaching the species barrier. These changes have occurred on almost every continent. They are exemplified by zoonotic diseases such as Lassa fever, first identified in West Africa in 1969 and now known to be transmitted to humans from human food supplies contaminated with the urine of rats that are in search of food, as their natural habitat could no longer support their needs.

In Latin America, Chagas disease emerged as an important human disease after mismanagement of deforested land caused triatomine populations to move from their wild natural hosts to involve human beings and domestic animals in the transmission cycle, eventually transforming the disease into an urban infection that can be transmitted by blood transfusion. Other zoonotic diseases that are increasing because of increased contact between people and naturally infected animal hosts include Lyme borreliosis in Europe and North America, transmitted to humans who come into contact with ticks that normally feed on rodents and deer, the reservoir of *Borrelia burgdorferi* in nature; and the Hantavirus pulmonary syndrome in Southwest North America.

The narrow band of desert in sub-Saharan Africa, in which epidemic *Neisseria meningitidis* infections traditionally occur, has enlarged as drought spread south, so that Uganda and Tanzania experience epidemic meningitis, while outbreaks of malaria and other vector-borne diseases have been linked to the cutting of the rainforests. In 1998 an outbreak of Japanese encephalitis in Papua New Guinea was linked to an extensive drought, which led to increased mosquito breeding as rivers dried into stagnant pools. The virus is now widespread in Papua New Guinea and other parts of Asia and threatening to move farther east. Buruli ulcer, a poorly understood mycobacterial disease that has emerged dramatically over the past decade, has erupted following significant environmental disturbances, and some evidence suggests that recent increases in Africa are linked to deforestation and subsequent flooding, or to the construction of dams and irrigation systems.

Finally, human behavior has played a role in the emergence and reemergence of infectious diseases, best exemplified by the increase in gonorrhea and syphilis during the late 1970s, and the emergence and amplification of HIV worldwide, which are directly linked to unsafe sexual practices and intravenous drug abuse. Human behavior has also facilitated the relentless evolution of anti-infective drug resistance. The mechanisms of resistance, a natural defense of microorganisms exposed to antimicrobial drugs, include both spontaneous mutation and genetic transfer. The selection and spread of resistant strains are facilitated by many factors, including human behavior in overprescribing drugs, in poor compliance, and in the unregulated sale of pharmaceuticals by nonhealth workers.

In Thailand, among 307 hospitalized patients, 36% who were treated with antiinfective drugs did not have an infectious disease. The overprescribing of antimicrobials occurs in most other countries as well. In Canada, it has been estimated that of the more than 26 million people treated with anti-infective drugs, 50% were treated inappropriately. Findings from community surveys of *E. coli* in the stool samples of healthy children in China, Venezuela, and the United States suggest that although multiresistant strains were present in each country, they were more widespread in Venezuela and China, countries where less control is maintained over antibiotic prescribing.

Animal husbandry and agriculture use large amounts of antimicrobials, and results in the selection of resistant bacterial strains in animals, which then genetically transfer the resistance factors to human pathogens or infect humans as zoonotic diseases, is a confounding factor that requires better understanding. Direct evidence exists that four multiresistant bacteria infecting humans, *Salmonella, Campylobacter, Enterococci*, and *E. coli*, are directly linked to resistant organisms in animals.

Globalization

Infectious diseases emerge and reemerge in a world where international travel facilitates their spread. Though the role of travel in the spread of infectious diseases has been known for centuries, the speed of such travel has increased during the past 50 years. Today, a traveller can be in an European or Latin American capital one day and be in the center of Africa or Asia the next day. With them, humans often carry infectious agents, many times without knowledge of infection because it is still in its incubation period. Like insects, humans have become important vectors of diseases in a globalized world.

During the 1990s, over 500 million people traveled by air each year, and contributed to the growing risk of exporting or importing infection or drug-resistant organisms. In 1988, a clone of multiresistant *Streptococcus pneumoniae* first isolated in Spain was later identified in Iceland. Another clone of multiresistant *S. pneumoniae*, also first identified in Spain, was subsequently found in the United States, Mexico, Portugal, France, Croatia, Republic of Korea, and South Africa. A study conducted by the Ministry of Health of Thailand on 411 existing tourists showed that 11% had an acute infectious disease, mostly diarrheal, but also respiratory infections, malaria, hepatitis, and gonorrhea.

By 2006, international travel had increased to over 2 billion, and had greatly increased the ease with which microbes, incubating in unsuspecting humans, can cross continents and invade new geographic territories. Microbes living in insects concealed in cargoes or in the luggage holds and cabins of jets also take the same pathway. In the late 1990s West Nile fever arrived in North America through the introduction of a single virus subtype, and today has become endemic in avian populations throughout the United States and in Southern Canada and Northern Mexico.

Once established on new continents, emerging or reemerging infectious diseases can change population dynamics and negatively impact on economies. Nothing more clearly demonstrates this global threat than the spread of AIDS in humans throughout the world during the latter half of the twentieth century. AIDS has had a negative impact on economic development and healthy population growth. In recent years, every continent has experienced an unexpected outbreak of some infectious disease directly related to increased travel, one of the most recent having been SARS in 2003 which had a negative impact on travel and trade throughout Asia.

Parallel increases in trade have also facilitated the international spread of microbes, in animals traded internationally, or in improperly or nonprocessed food and food products. As a result, the threat of epidemic diseases with origins in one country and spread to others has become a real and constant threat. Trade is the reason that BSE in cattle has been found in 24 countries in which cattle and/or cattle products including animal feed were traded. Rift Valley fever is thought to have arrived in the Arabian peninsula in infected livestock traded across the Red Sea from Eastern Africa, and it has now become endemic in these new geographic areas, adding to the infectious disease burden.

Advances in food production and storage technology, coupled with the globalization of markets, have resulted in a food chain that is unprecedented in its length and complexity, thus creating an efficient vehicle for microbes to spread to new areas and susceptible hosts. Tracing the origin of all ingredients in a meal has become virtually impossible, constituting an enormous challenge for the control of foodborne diseases.

The universal nature of the microbial threat, with agents of disease, including drug-resistant forms, passing undetected across increasingly porous borders, has placed all nations on an equally vulnerable footing. Economic prosperity has produced a world that is interconnected in matters of economics and trade, with the result that health has become both a domestic issue, and an issue with foreign policy considerations as well (**Table 2**).

Solutions

Attempts at regulation to prevent the spread of infectious diseases were first recorded in 1377 in quarantine legislation to protect the city of Venice from plague-carrying rats on ships from foreign ports. Similar legislation in Europe, and later the Americas and other regions, led to the first international sanitary conference in 1851, which laid down a principle for protection against the

Table 2 Resistance of common infectious diseases to antiinfective drugs, 1998

Disease	*Antiinfective drug*	*Range (%)*
Acute respiratory infection (*S. pneumoniae*)	Penicillin	12–55
Diarrhea (*Shigella*)	Ampicilline	10–90
	Trimethoprim	
	Sulfamethoxazole	9–95
Gonorrhea (*N. gonorrhoeal*)	Penicillin	5–98
Malaria	Chloroquine	4–97
Tuberculosis	Rifampicin	2–40
	Isonizid	
	Fluoroquinolone	
	Amikacin	
	Capreomycin	
	Kanamycin	

Source: WHO.

international spread of infectious diseases: maximum protection with minimum restriction. Uniform quarantine measures were determined at that time, but a full century elapsed, with multiple regional and interregional initiatives, before the International Sanitary Regulations were adopted in 1951. These were amended in 1969 to become the International Health Regulations (IHR), which are implemented by the World Health Organization (WHO).

The IHR (1969) provided a universal code of practice, which ranged from strong national disease detection systems and measures of prevention and control including vaccination, disinfection, and deratting. They initially required the reporting of four infectious diseases – cholera, plague, smallpox, and yellow fever. But when these diseases were reported, the regulations were often misapplied, resulting in the disruption of international travel and trade, and huge economic losses. For example, when the cholera pandemic reached Peru in 1991, it was immediately reported to WHO. In addition to its enormous public health impact, however, misapplication of the regulations caused a severe loss in trade (due to concerns for food safety) and travel, which has been estimated as high as $770 million. In 1994, an outbreak of plague occurred in India with approximately 1000 presumptive cases. The appearance of pneumonic plague resulted in thousands of Indians fleeing from the outbreak area, risking spread of the disease to new areas. Plague did not spread, but the outbreak led to tremendous economic disruption and concern worldwide, compounded by misinterpretation and misapplication of the IHR (1969).

A further problem with the regulations was that many infectious diseases, including those that are new or reemerging, were not covered even though they have great potential for international spread. These ranged from relatively infrequent diseases such as viral hemorrhagic fevers to the more common threat of meningococcal meningitis. Because of the problematic application and disease coverage of the IHR (1969), they have been revised and updated to make them more applicable to infection control and other public health menaces in the twenty-first century.

The revised IHR (2005) give a clear definition of what constitutes a public health emergency of international concern, thus helping countries to avoid inappropriate reactions to strictly localized events. They include unambiguous mechanisms for confidential collaboration between the affected country and WHO to verify the presence or absence of a suspected outbreak. For example, any event that may constitute a global public health threat is assessed by the country in which it is occurring using a decision instrument and, if certain criteria are met, an official notification must be provided to WHO. Notification is mandatory for a single case of human influenza caused by a new virus, poliomyelitis caused by a wild-type poliovirus, smallpox, and SARS. Potential threats are, however, no longer confined to a list of four communicable diseases. They include all public health emergencies of international concern, including those caused by other infectious agents, chemical agents, radioactive materials, and contaminated food.

To allow for a more sensitive and proactive system of detecting events and outbreaks, the IHR (2005) provide for a fundamental change in surveillance practices to include the use of unofficial information sources, such as the press or electronic media. The IHR (2005) are designed to become a true global alert and response system to ensure maximum protection against the international spread of diseases and other public health emergencies with minimum interference with trade and travel.

Finally, the IHR (2005) require the development of a core set of surveillance and control capacities in each country, and countries will be monitored by WHO to ensure that these capacities have been established. These capacities, along with efforts to minimize the impact of natural and anthropogenic changes in the environment, improved water and sanitation; effective communication of information about the prevention of infectious diseases; and more rationally prescribed use of antimicrobial drugs will help rebuild the weakened public health infrastructure. The challenge in the twenty-first century will be to continue to provide resources to strengthen and ensure more cost-effective infectious disease control, and implementation of the IHR (2005), while also providing the additional resources needed for other emerging public health problems such as those related to lifestyle and aging.

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Relevant Websites

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http://www.geis.fhp.osd.mil/ – DoD-GEISWeb, Global Emerging Infections System

http://www.idsociety.org/ – IDSA, Infectious Diseases Society of America

http://www3.niaid.nih.gov/ – National Institute of Allergy and Infectious Diseases, National Institutes of Health

http://depts.washington.edu/ – UW Departments Web Server

Endosymbionts and Intracellular Parasites

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Defining Statement
Chronic Microbial Infections of Eukaryotes
Functional Significance of Endosymbionts
Transmission of Microbial Symbionts and Parasites
Persistence of Associations
Further Reading

Glossary

chronic infection Persistent infection by microorganisms.

horizontal transmission Acquisition of microorganisms from the environment or a host other than the parent, with the consequence that the microorganisms in parent and offspring hosts are not necessarily related.

symbiosis The intimate association between phylogenetically different organisms, often restricted to relationships from which all organisms derive benefit.

symbiosomal membrane Membrane of host origin bounding an intracellular symbiotic microorganism.

vertical transmission The transmission of microorganisms from a parent to offspring host.

Abbreviation

ROS	reactive oxygen species

Defining Statement

Eukaryotes bear persistent (i.e., chronic) microbial infections. Most microorganisms are not deleterious to the eukaryotic host, and some are beneficial or even required by the host. Many mutualistic microorganisms expand the metabolic repertoire of their host or are beneficial through effects that cannot be attributed to defined microbial traits.

Chronic Microbial Infections of Eukaryotes

Eukaryotes ('hosts') provide multiple habitats for proliferating populations of microorganisms, including representatives of the eubacteria, archaea, protists, and fungi. In other words, eukaryotes bear chronic (i.e., persistent) infections with microorganisms that generally are not deleterious and, in some cases, are beneficial or even required by the host. To illustrate, more than 90% of the cells and an estimated 99% of all genes in a healthy human are microbial. Only a minority of microorganisms associated with most eukaryotes are pathogens.

The relationships are most conveniently classified by two complementary criteria: their impact on the eukaryotic host and their location.

Impact on the Eukaryotic Host

By convention, the relationships between microorganisms and their eukaryotic hosts are assigned to three categories: mutualism, where the host benefits; commensalism, where the microorganism has no observable effect on host fitness; and parasitism, where the microorganism is deleterious to the host. Despite its widespread use, this classification is recognized to be artificial. The effect of a microorganism on eukaryotes can vary with host species, genotype, developmental age and condition, and with environmental circumstance. For example, opportunistic pathogens have no detectable impact on healthy hosts but cause disease in immunocompromised or otherwise debilitated hosts. Similarly, mycorrhizal fungi associated with plant roots are generally beneficial to plants by enhancing plant mineral nutrition, but their demand for plant-derived photosynthetic carbon can be deleterious to young seedlings with limited photosynthetic capacity.

A further term, symbiosis, is used widely to describe associations between microorganisms and their hosts. Some authorities regard symbiosis as synonymous with mutualism, while others subscribe to the original definition of symbiosis as 'any association between differently named organisms'. Similarly, the term 'symbiont' is interpreted by some to describe mutualistic microorganisms (as distinct from commensal and parasitic microorganisms) and by others to mean any microorganism in a eukaryotic host. The more general definition of symbiosis and symbiont is used extensively in the symbiosis literature but very rarely in the parasitology literature, and overt parasites are rarely considered as symbiotic. For this reason, this article refers to symbionts as microorganisms, which are generally advantageous to their host.

Immediately relevant to the definition of symbiont are symbiont-derived organelles in eukaryotes. As the term implies, these organelles have evolved from intracellular symbionts. A symbiont-derived organelle is characterized by two linked traits: first, genes ancestrally in the symbiont have been transferred to the host nucleus; second, the cognate proteins are targeted back to the organelle. In this way, the symbiont-derived organelle is inextricably dependent on the host lineage in which it evolved. There is overwhelming evidence for a symbiotic origin of mitochondria and plastids, both of which retain small numbers of genes.

Location in Eukaryotic Host

Microorganisms living within their hosts are termed endosymbionts (and endoparasites), as distinct from ectosymbionts (and ectoparasites), which are located on the surface of the host. Endosymbionts are classified as either intracellular (within cells) or extracellular (external to cells). Within multicellular hosts, the extracellular symbionts may reside in cavities or other spaces, for example, the body cavity and gut of animals, or between closely apposed host cells, for example, endophytic and mycorrhizal fungi in plant shoots and roots, respectively, and the latter are sometimes referred to as ‘intercellular’. Most intracellular microorganisms are restricted to the cytoplasm and separated from the cytoplasmic contents by a membrane of host origin, variously known as the symbiosomal membrane or parasitiphorous vacuole. Exceptionally, some intracellular symbionts and parasites lie free in the host cell cytoplasm, for example, the γ-proteobacterium *Wigglesworthia* symbiont in tsetse fly, or are localized to specific organelles, for example, rickettsias in mitochondria of the tick *Ixodes*, various bacteria in the nucleus of ciliates.

Although a broad phylogenetic range of microorganisms adopt the intracellular lifestyle, the diversity of intracellular microorganisms in any one host is low, generally one to several taxa. A far higher diversity of microorganisms colonize extracellular habitats in eukaryotic hosts. In particular, the guts of many animals bear very diverse microbial communities, comprising hundreds of taxa and including both ‘resident’ species that persist for substantial periods up to the full lifespan of the host and ‘transients’ that pass through the digestive tract with food. Among vertebrate animals, the only known intracellular microorganisms are parasites. The reasons for the apparent absence of intracellular symbionts are unknown, but may be related to the adaptive immune system of these animals.

Functional Significance of Endosymbionts

Symbioses as a Source of Metabolic Capabilities

Many endosymbioses are founded on the fact that microorganisms have a wider metabolic repertoire than their hosts. In particular, the lineage giving rise to the eukaryotes lacked the key metabolic capabilities of aerobic respiration, photosynthesis, and nitrogen fixation. Various eukaryotic groups also lack additional capabilities; for example, some protists and all animals cannot synthesize 9 of the 20 amino acids that contribute to protein (the ‘essential’ amino acids) and some coenzymes required for functioning of key enzymes (vitamins); the insects additionally cannot synthesize sterols *de novo*, and vertebrates cannot degrade cellulose. Repeatedly, eukaryotes have gained access to these metabolic capabilities by forming symbioses with microorganisms. Eight key metabolic capabilities of eukaryotes have a symbiotic basis, and an overview of these is provided in **Table 1** and the following text.

Aerobic respiration

Eukaryotes have acquired aerobic respiration from just one lineage of microorganisms, an α-proteobacterium allied to rickettsias, which evolved into mitochondria. The evidence is that all mitochondria are autonomous organelles (i.e., a cell line from which mitochondria have been eliminated cannot resynthesize these organelles) and possess coding DNA with unambiguous sequence similarity to rickettsias. Various genes derived from the mitochondrial ancestor have been transferred to the nucleus of the eukaryotic host and are present in the nucleus of taxa lacking mitochondria (e.g., trichomonads), leading to the current consensus that the ancestor of all modern eukaryotes was mitochondriate. It is disputed whether the acquisition of mitochondria predated (and was perhaps instrumental in) the evolutionary origin of eukaryotes, or occurred later, in a host bearing the key eukaryotic trait of a membrane-bound nucleus.

Photosynthesis

Oxygenic photosynthesis evolved once, in the ancestor of the cyanobacteria, and has been acquired multiply by eukaryotes. By far, the most widespread and important photosynthetic symbiont is the cyanobacterial lineage that evolved into plastids. Phylogenetic analyses point firmly to a single evolutionary origin of plastids, acquired by the protist ancestor of chlorophytes (which gave rise to the land plants), rhodophytes (red

Table 1 An overview of endosymbioses that enhance the metabolic repertoire of eukaryotes

Metabolic capability	*Examples of endosymbioses*
Aerobic respiration	Mitochondria (evolved from α-proteobacteria) in most eukaryotes
Oxygenic photosynthesis	Plastids (evolved from cyanobacteria) in algae and plants. Cyanobacteria (e.g., *Nostoc*) and algae (e.g., *Trebouxia*) in lichenized fungi. Various algae (e.g., freshwater *Chlorella* and marine *Symbiodinium* in protists and animals (e.g., corals)
Chemoautotrophy	Various bacteria in marine animals, e.g., Pogonophora (e.g., *Riftia*) and bivalves
Nitrogen fixation	Rhizobia in legumes, *Frankia* (actinomycete) in various dicot plants,[a] cyanobacteria in some lichenized fungi, plants (e.g., *Gunnera*, *Azolla*, cycads) and diatoms (e.g., *Rhizosolenia*). Various bacteria in termites, teredinid mollusks
Essential nutrient provision (e.g., essential amino acids, vitamins, and sterols)	Various bacteria and fungi in protists and animals, especially insects feeding on vertebrate blood, plant sap, and wood
Cellulose degradation	Various bacteria (e.g., *Ruminococcus*) in herbivorous vertebrates, for example, ruminants; protists in lower termites
Methanogenesis	Methanogenic bacteria associated with anaerobic ciliates
Bioluminescence	Bacteria (*Vibrio*, *Photobacterium*) in marine teleost fish and squid
Secondary metabolites as protective toxins	Bacteria in various animals, endophytic fungi in grasses

[a] Members of eight families: Betulaceae, Casuarinaceae, Coriariaceae, Dastiscaceae, Eleaginaceae, Myricaceae, Rhamnaceae, and Rosaceae.

algae), and a small third group of algae, the glaucophytes. Representatives of chlorophytes and rhodophytes have been acquired by other protists, giving rise to additional algal groups bearing complex plastids bounded by multiple membranes (**Figure 1**). Some host lineages have subsequently become nonphotosynthetic but retained their erstwhile plastids, which perform different but essential functions. For example, the apicomplexan protists bear an organelle, the apicoplast, required for its capacity to synthesize essential terpenoids, and the genome of which is unambiguously allied to the genome of chromophyte plastids.

Various photosynthetic cyanobacteria and algae enter into symbioses with nonphotosynthetic hosts. Of particular importance are the lichens, associations of fungi with algae or cyanobacteria, which dominate large areas of tundra and are abundant in temperate and tropical forests. An estimated 14 000 fungal species, including nearly half of all described ascomycetes, are lichenized, and the symbionts include 30–40 genera of algae, mostly chlorophytes, and at least 12 genera of cyanobacteria. In the marine environment, the symbiosis between corals and dinoflagellate algae of the genus *Symbiodinium* is the architectural foundation of shallow-water coral reefs, which are highly productive and diverse ecosystems of immense ecological and socioeconomic importance.

Chemoautotrophy

Chemoautotrophic bacteria fix carbon dioxide using the energy and the reductant derived from the oxidation of reduced (usually inorganic) compounds, generally with molecular oxygen as the electron acceptor. Various chemoautotrophs form symbioses with animals living at the interface between oxic and anoxic environments, representing a source of oxygen and reduced substrate, respectively. Habitats include the zone in marine sediments where oxygen-rich seawater percolating downward meets anoxic sediment water, deep-sea hydrothermal vents, natural gas and methane seeps, and the immediate environment around sewage outfalls. Most symbiotic chemoautotrophs are sulfur oxidizers or methane oxidizers. Their animal hosts include bivalves, pogonophoran worms, nematodes, and annelids, and these symbioses are particularly conspicuous at hydrothermal vents, where the hosts include vestimentiferan tube worms, such as *Riftia*, up to 2 m in length, and very large bivalves, such as *Calyptogena* species.

A recent metagenomic analysis of the symbiosis in the interstitial oligochaete annelid *Olavius algarvensis* has revealed microbial complexity. This symbiosis involves four bacterial taxa located internal to the host body wall cuticle: γ1-, γ3-, δ1-, and δ4-proteobacteria. Shotgun sequencing, gene identification, and metabolic reconstruction *in silico* indicated that the γ-proteobacteria are sulfide-oxidizing chemoautotrophs and that the δ-proteobacteria are sulfate-reducing bacteria that can also fix carbon dioxide by the reductive acetyl coenzyme A pathway and the TCA cycle. The complementary pathways of sulfate reduction and sulfide oxidation provide for a symbiotic sulfur cycle, ensuring that the γ-proteobacteria are provided with sulfide, even though the external habitat has no detectable sulfide. The animal host lacks any gut, mouth, or anus and is believed to gain most of its nourishment by engulfing and digesting the subcuticular bacteria. The bacteria also have the genetic capacity to consume host waste ammonia, and this may explain the reduced nephridial excretory system in this species.

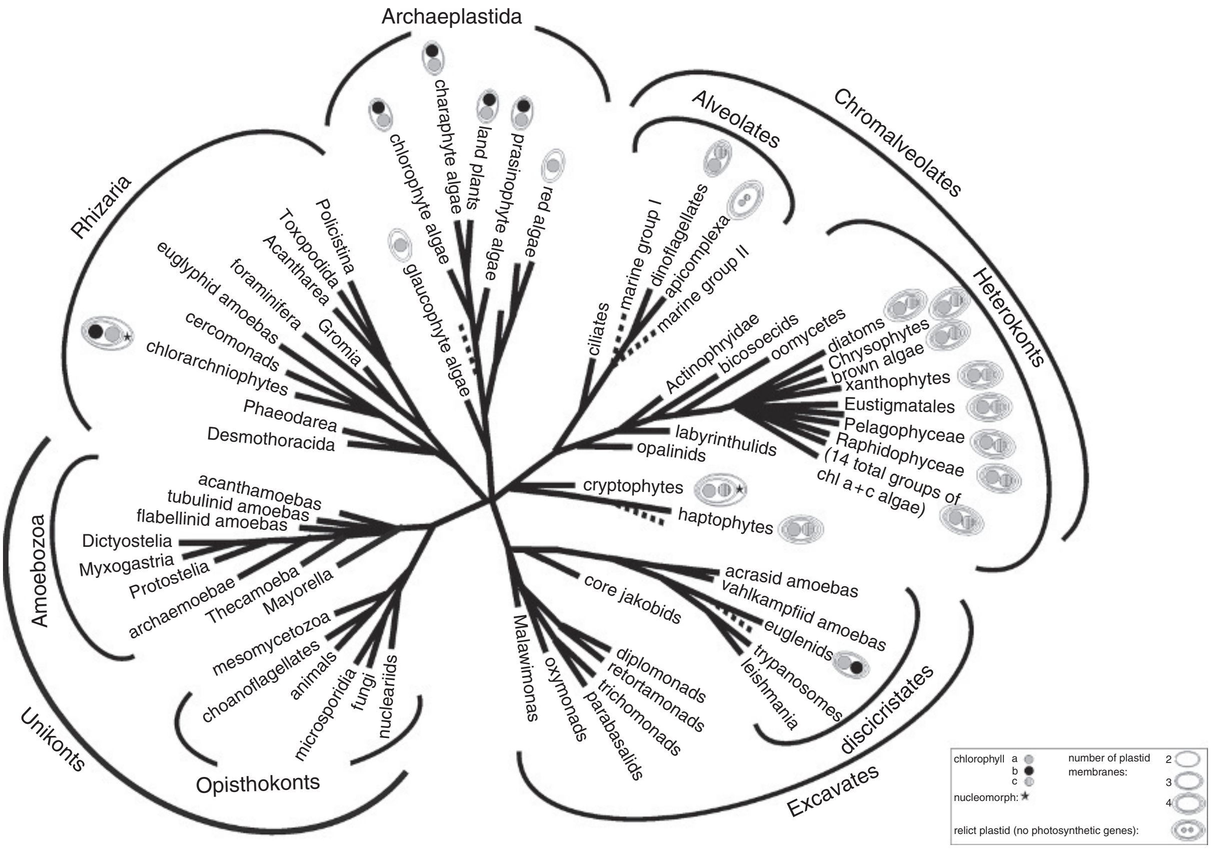
Archaeplastida
charaphyte algae
land plants
prasinophyte algae
red algae
chlorophyte algae
glaucophyte algae
Alveolates
Chromalveolates
marine group I
dinoflagellates
apicomplexa
marine group II
ciliates
Heterokonts
Actinophryidae
bicosoecids
oomycetes
diatoms
Chrysophytes
brown algae
xanthophytes
Eustigmatales
Pelagophyceae
Raphidophyceae
(14 total groups of chl a+c algae)
labyrinthulids
opalinids
cryptophytes
haptophytes
Rhizaria
Policistina
Toxopodida
Acantharea
Gromia
euglyphid amoebas
foraminifera
cercomonads
chlorarchniophytes
Phaeodarea
Desmothoracida
Amoebozoa
acanthamoebas
tubulinid amoebas
flabellinid amoebas
Dictyostelia
Myxogastria
Protostelia
archaemoebae
Thecamoeba
Mayorella
Unikonts
Opisthokonts
mesomycetozoa
choanoflagellates
animals
microsporidia
fungi
nucleariids
Malawimonas
oxymonads
core jakobids
diplomonads
retortamonads
trichomonads
parabasalids
acrasid amoebas
vahlkampfiid amoebas
euglenids
trypanosomes
leishmania
discicristates
Excavates
chlorophyll a ●
b ●
c ●
nucleomorph: ★
number of plastid membranes:
2
3
4
relict plastid (no photosynthetic genes):

Figure 1 The phylogenetic distribution of plastids in eukaryotes, mapped onto an unrooted phylogenetic tree of eukaryotes based on a combination of molecular phylogenetic and ultrastructural data. According to this evolutionary scenario, the diversity of plastids can be explained by three evolutionary events. (1) The cyanobacterial ancestor of all plastids was acquired by the ancestor of the archaeplastida, giving rise to three groups – the rhodophytes (red algae) and the glaucophytes containing chlorophyll *a* and the chlorophytes (green algae) and allies, including the land plants, containing chlorophyll *a* and *b*. (2) A rhodophyte alga was acquired by the ancestor of the chromalveolates, generating complex plastids that have chlorophyll *a* and *c*. (3) A chlorophyte was acquired by the ancestor of the euglenids and the chlorarachniophytes. Among the complex plastids generated through steps (2) and (3), the nucleus of the primary host has been retained as a nucleomorph in the chlorarachniophytes and one group of chromalveolates, the cryptophytes. Reproduced from Baldauf S. L. (2003). The deep roots of eukaryotes. *Science* 300: 1703–1706.

Nitrogen fixation

The phylogenetic distribution of nitrogen fixation is broad but irregular, indicative of multiple evolutionary acquisitions and losses. The lateral transfer of nitrogen fixation genes has apparently been widespread among bacteria, but eukaryotes have gained this capability exclusively by symbiosis with bacteria, none of which (to our knowledge) have evolved into organelles.

The nitrogen-fixing endosymbionts are best known in plants. Of particular importance are the nitrogen-fixing bacteria in the root nodules of legumes (comprising two separate lineages of α-proteobacteria, one including *Bradyrhizobium* and *Azorhizobium* and the other including *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, and β-proteobacteria of the genera *Burkholderia* and *Ralstonia*) and the actinomycete *Frankia* in various dicot plants. An estimated 150 species of vascular plants, including many cycads, the water fern *Azolla*, and the dicot *Gunnera*, associate with cyanobacterial symbionts of the family Nostocaceae that comprise filaments of vegetative cells (capable of oxygenic photosynthesis) and heterocysts (which fix nitrogen), and the chief advantage to the plant host is access to fixed nitrogen derived from the heterocysts. In addition, an estimated 550 species of lichenized fungi associate with both photosynthetic algae and cyanobacteria restricted to specialized structures called cephalodia, in which they fix nitrogen at high rates. These cyanobacterial symbionts are the only known nitrogen-fixing symbioses in fungal hosts.

Nitrogen-fixing bacteria are present in the gut microbiota of many animals, but they generally are at low abundance and of no nutritional significance to the animal host. Some animals feeding on nitrogen-poor wood (e.g., some termites, *Tenebrio* shipworms) gain nitrogen from nitrogen-fixing bacterial endosymbionts. There is a general expectation that nitrogen-fixing symbioses are less significant in animals than in plants because animals have a limited capacity to utilize the primary nitrogen fixation product, ammonia. Indeed, ammonia is a potentially toxic waste product of metabolism for most animals.

Provision of essential nutrients

Most research on microorganisms that provide specific classes of primary nutrients, such as amino acids and vitamins, has focused on intracellular endosymbionts of insects that are restricted to specific organs, variously known as mycetomes or bacteriomes (**Table 2**). Although these symbioses have evolved independently multiple times between diverse groups of insects and microorganisms, the associations have three common traits. (1) The microorganisms are restricted to specific insect cells, the sole function of which appears to be to house and maintain the microorganisms; these cells are known as bacteriocytes or mycetocytes (the terms are synonymous), forming organs known as bacteriomes or mycetomes. (2) The microorganisms are obligately vertically transmitted, usually by insertion from the maternal bacteriocytes directly into the eggs in the female ovary. (3) The association is required by both insect and microorganisms.

The anatomical location and structural organization of the bacteriome vary widely; the bacteriome may be associated with the animal gut (e.g., tsetse flies), fat body (e.g., cockroach), Malpighian tubules (e.g., book lice), or lie free in the body cavity (e.g., aphids). The microorganisms are restricted to the cytoplasm. They are generally unculturable and molecular methods have revealed their diversity, including α-, β-, and γ-proteobacteria, flavobacteria, and fungi, some of which are not closely related to any formally described taxa. Where similar or identical sequences are identified in multiple host species of one order or family, the endosymbiont has been assigned a novel candidate generic name (**Table 2**).

The nature of the symbiont–host interactions has been inferred from the distribution of these relationships, which are particularly prevalent in insects feeding on nutrient-poor diets, such as plant sap (phloem or xylem) deficient in essential amino acids and vertebrate blood deficient in B vitamins (**Table 2**). Direct experimental evidence for the translocation of essential amino acids from bacteria to insects has been obtained for cockroaches and aphids, and the nutritional role of other symbiotic microorganisms is inferred from their complement of metabolic genes, identified either by PCR amplification or from completely sequenced genomes.

Microsopical studies have revealed various other animals, usually invertebrates including earthworms, leeches, tunicates, and nematodes, which universally bear dense aggregations of microorganisms, in specific anatomical

Table 2 Distribution of intracellular microbial endosymbioses in insects

Insect	*Microorganisms*
(a) plant sap feeders	
Heteroptera	
Plataspids (stinkbugs)	*Ishikawella* (γ-proteobacteria)
Alydids (broad-headed bugs)	*Burkolderia* (β-proteobacteria)
Homoptera	
Auchenorrhyncha (including leafhoppers, planthoppers, cicadas)	*Baumannia cicadellinicola* (γ-proteobacteria) and *Sulcia muelleri* (Bacteroidetes); Pyrenomycete fungi in some planthoppers
Aphids	*Buchnera* (γ-proteobacteria) or pyrenomycete fungi
Whitefly	*Portiera aleyrodidarum* (γ-proteobacteria)
Psyllid jumping lice	*Carsonella ruddii* (γ-proteobacteria)
Scale insects & mealy bugs)	*Tremblaya princes* (β-proteobacteria)
(b) vertebrate blood	
Heteroptera	
Cimicid (bedbugs)	γ-proteobacterium allied to *Serratia*
Triatome bugs	*Arsenophonus triatominarum* (γ-proteobacteria)
Anoplura (sucking lice)	*Riesia pediculicola* ((γ-proteobacteria) in human head louse & body louse
Diptera Pupiparia	*Wigglesworthia* in *Glossina*
	Arsenophonus in streblids and hippoboscids
(c) general feeders	
Blattidae (cockroaches)	*Blattabacterium* (flavibacteria)
Mallophaga (biting lice)	Not known
Psocoptera (book lice)	*Rickettsia* sp.
Beetles, e.g.	
Weevils	Various γ-proteobacteria
Anobiid timber beetles	*Symbiotaphrina* (yeasts)
Hymenoptera	
Camponoti (carpenter ants)	*Blochmannia* (γ-proteobacteria)

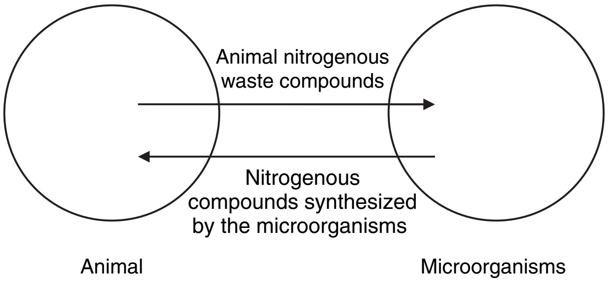
Animal nitrogenous waste compounds
Nitrogenous compounds synthesized by the microorganisms
Animal
Microorganisms

Figure 2 Nitrogen recycling by endosymbiotic microorganisms. The microorganisms (collectively displayed here as a circle) transform nitrogenous waste products of the animal (ammonia, urea, etc.) into nitrogenous compounds valuable to animal metabolism, and these compounds are translocated back to the animal tissues.

locations. Similarly, some protist species consistently bear intracellular bacteria. The identity and function of the microorganisms have received little study, but a nutritional role is often invoked. Genome sequence analysis is assisting with the construction of specific hypotheses. For example, the bacteria *Wolbachia* in filarial nematodes have been implicated in the provision of nucleotides and heme to their host on the basis of the predicted gene complement of the complete genome sequence.

Immediately related to the nutrient provisioning is the role of microorganisms in recycling, especially of nitrogen. Nitrogen recycling refers to the microbial consumption of animal waste nitrogenous compounds (e.g., ammonia and urea) to synthesize 'high-value' nitrogenous compounds (e.g., essential amino acids), which are released back to the host (**Figure 2**). Nitrogen recycling has been implicated from radiotracer and ^{15}N-tracer studies of several symbioses, including cockroach–flavobacteria and the relationship between corals and their dinoflagellate algal symbionts *Symbiodinium.*

Cellulose degradation

Vertebrate animals lack the capacity to digest cellulose and other plant cell wall polysaccharides, such as hemicellulose, and many herbivores can exploit these compounds only by association with cellulolytic microorganisms. Typically, the microorganisms are restricted to an anoxic portion of the gut, the 'fermentation chamber' where they degrade the plant polymers to support their own growth, releasing short-chain fatty acids as the waste products of anaerobic respiration. These compounds diffuse into the host bloodstream and are used as substrates for aerobic respiration by the animal host.

These symbioses are known in the hindgut (colon or cecum) of virtually all herbivorous mammals (the giant panda is reputedly an exception) and various herbivorous birds and lizards. They are called postgastric symbioses because the fermentation chamber is distal to the enzymatic region of the gastrointestinal tract. Some mammals, for example, ruminants, such as cattle, sheep, deer, kangaroos, and colobine monkeys, and at least one bird, the leaf-eating hoatzin, additionally have pregastric fermentation chambers, that is, proximal to the digestive region. Cellulolytic symbioses are apparently rare among invertebrate animals, probably because many invertebrates have intrinsic cellulases and because, for small animals, the costs of maintaining an anoxic fermentation chamber would be unduly high. However, a minority of wood-feeding termites exploit cellulolytic microorganisms in an enlarged hindgut, known as the paunch. Unlike the bacterial cellulose-degrading symbionts of vertebrates, the cellulolytic symbionts in termites are obligately anaerobic flagellate protists of the orders Hypermastigida, Trichomonadida, and Oxymanidida. At least 400 species have been reported, most unknown from any other location, and several, including *Trichomitopsis termopsidus* and *Trichomympha sphaerica*, have been brought into culture. Related protists occur in wood-eating roaches of the genus *Cryptocercus.*

The cellulolytic microorganisms represent one of many functional groups of microorganisms in the digestive tracts of many animals (see below). For the majority of associations that are postgastric, only diffusible microbial products (e.g., short-chain fatty acids) are available to the host. Other microbial products (proteins, vitamins, etc.) are lost from the system via the feces and are recovered only in host species that display coprophagy, that is, consumption of feces. Animals with pregastric fermentation chambers, by contrast, can gain nutrients from their microbiota by the digestion of microbial cells or other products that pass from the fermentation chamber into the gastric stomach.

Methanogenesis

Methanogenic bacteria generate ATP by synthesizing methane under strictly anoxic conditions, most commonly by the reduction of carbon dioxide with hydrogen. All known methanogens are euryarchaeote Archaea. The consumption of hydrogen by methanogens is advantageous to anaerobic eukaryotes because the rate of oxidative reactions, such as glycolysis, can otherwise be depressed by high levels of hydrogen. In other words, methanogens can act as an electron sink for anaerobic hosts.

Methanogenic symbioses are prevalent in anaerobic ciliate protists. Phylogenetic analyses suggest that the symbiotic habit has evolved multiple times among methanogens and that most symbionts are closely related to free-living taxa. The symbiotic methanogens may be ectosymbiotic, for example, on the surface of ciliates of the family Entodiniomorphida living in the rumen of cattle, or intracellular, as for *Methanobacterium formicicum* in *Plagiopyla* and *Metopus* species in rice paddies and landfill sites, and *Methanobrevibacter* species in cellulolytic protists in termite guts.

Bioluminescence

Luminescence is the generation of light by the oxidation of a substrate, generically known as luciferin, catalyzed by the enzyme luciferase. Bioluminescence has evolved at least 35 in eukaryotes, involving at least 5 different biochemical reactions, and is rare in bacteria, being restricted to four genera, *Vibrio*, *Photobacterium*, *Alteromonas*, and *Xenorhabdus*. Linked to this, most instances of luminescence in eukaryotes are intrinsic; the only known luminescent symbioses involve *Vibrio/Photobacterium* in a minority of marine teleost fish and squid, and *Xenorhabdus* in some terrestrial entomopathogenic nematodes (see 'Synthesis of secondary compounds').

Marine animals use light for communication in shoaling and courtship (e.g., flashlight fish), as a startle response to distract potential predators in deep waters, as a lure (e.g., angler fish) and as camouflage (by counterillumination to obscure the silhouette of the animal otherwise evident against downwelling light to predators lower in the water column). The luminescent symbioses in marine fish and squid are housed in light organs. The symbiotic bacteria are restricted to many narrow tubules, with access to the nutrients and oxygen required for sustained light production. They are maintained at high densities; this is essential for light production, which is regulated by quorum sensing via an autoinducer that, on reaching a certain concentration, induces the expression of the *lux* genes coding for the luciferase. Bacterial luminescence is generally less intense than intrinsic sources, requiring mirrors and lenses to maximize emission, and, unlike intrinsic luminescence, it is continuous (it cannot be turned off), requiring shutters, chromatophores, and so on to control the timing of its emission. Essentially, the greater anatomical complexity of symbiotic light organs than intrinsic light organs relates to the limitations of bacteria as a light source.

Synthesis of secondary compounds

Various eukaryotic groups have exploited the capacity of microorganisms to synthesize bioactive secondary compounds, principally as mediators of antagonistic interactions with natural enemies or prey. One of the best-studied relationships is between grasses, particularly agronomically important species, and endophytic fungi of the form genus *Neotyphodium* allied to the genus *Epichloë*. Hyphae of these fungi, which ramify through the shoot of the grass, contain alkaloids that are toxic to animals. Animals that feed on fungal-infected grasses ingest these alkaloids and become debilitated and may die. For example, ergot and indole diterpene-type alkaloids present in endophytes of tall fescue and perennial ryegrass cause neurological disorders known as 'the staggers' in cattle and sheep and also confer resistance to various insect and nematode pests.

The incidence of animals that derive protection from secondary metabolites synthesized by symbiotic microorganisms is uncertain, and only a few examples have been explored in detail. These include the production of compounds known as 'bryostatins' by the bacterium *Endobugula sertula* in the marine bryozoan *Bugula neritina*; polyketides, such as pederin, by uncultured pseudomonads both in beetles of the genus *Pederus* and in sponges, including *Theonella swinhoei*; and a variety of cyclic patellamide peptides by cyanobacteria of the genus *Prochloron* in marine ascidians (sea squirts). All these compounds have been suggested to have a protective role against predators, and this has been demonstrated for the *Pederus* beetles. Furthermore, the genes for pederin synthesis are borne on a putative pathogenicity island in the pseudomonad genome, suggesting that this capability is horizontally transmissible among bacteria.

A further instance of secondary product biosynthesis by microbial symbionts is provided by the luminescent bacterium *Xenorhabdus luminescens*, the obligate symbiont of soil-borne heterorhaditid nematodes that parasitize insects. When the nematodes infect a susceptible insect, the bacteria are released into the insect hemolymph ('blood') and proliferate rapidly. In this location, they synthesize compounds, including antibiotics, thereby preventing invasion by other bacteria. This ensures that the insect habitat is sustained for the 2 weeks required for the reproduction of the nematodes. The bacteria also produce a red anthraquinone pigment and light, believed to attract new insect hosts by day or night toward the young infective nematodes as they emerge from the insect host. Some authorities, however, doubt the significance of the luminescence in this context because the light intensity is very low. An alternative possible role of the luciferase is as a terminal oxidase with greater affinity for oxygen than the cytochrome pathway, promoting aerobic metabolism at low oxygen tensions in the insect cadaver.

A remarkable instance of evolutionary change in function is provided by the protein GroEL synthesized by the symbiotic bacteria *Enterobacter aerogenes* in antlions (insects of the family Myrmeleontidae, a group of lacewings). GroEL is a bacterial chaperone that mediates protein folding, but the GroEL homologue in the symbiotic *E. aerogenes* is a potent toxin, released in the insect saliva that paralyzes prey.

Symbiont-Mediated Modification of Host Physiology and Vigor

Comparisons of the traits of various hosts bearing and experimentally deprived of their symbiotic microorganisms have revealed differences that cannot be attributed readily to defined traits of the microorganisms. For example, plants infected with mycorrhizal fungi are commonly more drought-tolerant than uninfected congeners, the gut microbiota of animals can confer resistance to gut pathogens, the tolerance of high temperatures by some insects is promoted by specific microbial symbionts, and calcification and skeletal growth of many corals is promoted by the presence of symbiotic dinoflagellate algae. In most instances, the underlying processes are obscure or uncertain, but microbial impacts on the host metabolism or hormonal and immune systems have been invoked. It should also be recognized that, although generally beneficial, these interactions can be deleterious to the host in certain circumstances. For example, the gut microbiota can promote obesity in mice and humans, and the commensal gut bacteria in insects are instrumental in the mortality of insects that ingest the δ-endotoxin of *Bacillus thuringiensis* (the toxin-mediated modification of the insect gut wall allows commensal gut microorganisms to gain entry into the insect body cavity, where they proliferate causing lethal septicemia).

Microbial-mediated modification of host traits has been studied in detail for one group of insects, the aphids. Many aphids bear one to several ‘secondary symbionts’ in addition to the obligate intracellular bacterium *Buchnera*, which has a required nutritional role. The secondary symbionts have a wider tissue distribution than *Buchnera* and can be acquired horizontally both by the oral route and sexually. The secondary symbionts are not required by the aphids and are absent from many individual aphids in natural populations. They are all bacteria and include a rickettsia, a *Serratia* species, recently assigned the candidate name *S. symbiotica*, and two further γ-proteobacteria with the candidate names *Regiella insecticola* and *Hamiltonella defensa* that are not closely related to any formally described taxa. *S. symbiotica* can ameliorate the negative impact of heat shock on aphid fecundity and survivorship, while *R. insecticola* promotes aphid resistance to entomopathogenic fungi and *H. defensa* can protect aphids from parasitoids. In the pea aphid, the prevalence of the secondary bacteria differs with the plant from which the aphids are isolated. For example, aphids on clover species generally bear *R. insecticola*, while those on alfalfa tend to have *H. defensa*. The contribution of the bacteria and aphid genotype to these patterns is uncertain because experiments on the impact of manipulating the secondary symbiont complement on the capacity of aphids to use plant species have yielded contradictory results.

Intracellular Parasites

The intracellular habitat has several plausible advantages for microorganisms: evasion of extracellular defenses (e.g., antimicrobial peptides, complement and circulating antigens in the blood and tissue fluids), access to an alternative source of nutrients, and as a route to colonize new tissues in multicellular hosts. It is exploited by various parasitic microorganisms: facultative intracellular parasites, which can also thrive in the extracellular condition (e.g., in blood or the tissue fluids of animals), and obligate intracellular parasites, which can proliferate only within eukaryotic cells. Facultative parasites include *Shigella* species, which proliferate and spread among epithelial cells of the colon, and *Mycobacterium tuberculosis*, which invades the alveolar macrophages of the lungs. Rickettsias, mycoplasmas, and *Chlamydia* species are all obligate intracellular parasites.

Most intracellular parasites are deleterious by causing disease, either through direct effects, for example, toxins, or as a consequence of the host immune response to their presence. One exceptional type of intracellular parasite is the reproductive parasites. These parasites are transmitted vertically via females and specifically target male hosts, for example, by killing or feminizing them or by inducing parthenogenesis in females (see below). Reproductive distortion by microorganisms is apparently restricted to arthropods and is mediated by very few microorganisms. Most known instances involve the α-proteobacterium *Wolbachia*, reportedly present in at least 20% of all insects, but *Cardinium* (Bacteroidetes), microsporidia, flavobacteria, and spiroplasmas have also been implicated.

Transmission of Microbial Symbionts and Parasites

Two modes of transmission are recognized. Vertical transmission is the transfer of microorganisms from parent to offspring, usually via the mother in sexually reproducing hosts. Horizontal transmission is the acquisition of microorganisms from the environment or a host other than the parent, with the consequence that the microorganisms in parent and offspring hosts are not necessarily related.

Vertical Transmission

Vertical transmission has important evolutionary consequences for microorganisms. The offspring of the host represent habitats for the progeny of vertically transmitted microorganisms, with the consequence that the microorganisms have a selective interest in the reproductive output of their host. Most vertically transmitted symbionts are, consequently, beneficial to their hosts, and vertical transmission can be considered as a route by which hosts ‘impose’ mutualistic traits on their microbial partners. Furthermore, where vertical transmission is obligate, the phylogenies of the host and microorganism are congruent.

The advantage to the host of vertical transmission is that its offspring are assured of gaining a compatible symbiont – or, at least, a symbiont compatible with its parent. Vertical transmission is particularly important where the symbionts are rare in the free-living environment. Vertical transmission may involve highly regulated, coordinated processes in host and microorganism. This is illustrated by the transmission of the bacteria *Riesia pediculicola* in the human body louse *Pediculus humanus*. The mycetocytes in this insect are aggregated together as a coherent organ, the stomach disc, just ventral to the stomach. During the final molt of the female insect, the bacteria are expelled from the mycetome and they migrate to the reproductive tract, such that in the adult female louse, all the bacteria are associated with the reproductive organs. In the male, they are retained in the stomach disc. The bacteria in the adult females are initially associated with the lateral oviducts. They subsequently penetrate the oviduct wall and gain access to the insect cells lining the oviduct. From here, they are transferred to the insect cells in the pedicel and then to the cytoplasm of each egg as it matures (**Figure 3**).

Although, as considered above, vertical transmission promotes overlap in the selective interests of the host and its complement of microorganisms, it does not eliminate conflict between the partners. In sexual hosts with exclusively maternal transmission, the source of conflict is the sex ratio of the host offspring, with a 1:1 female to male ratio generally optimal for the host, and an excess of females optimal for the microorganism; all microbial cells transmitted to a male host will die with that host. Reproductive distortion is widespread in arthropods, mediated by *Wolbachia* and other taxa in several different ways as follows:

1. Parthenogenesis, to give twice the number of female offspring relative to uninfected hosts, appears to be restricted to host taxa with haplodiploid sex determination (i.e., fertilized (diploid) eggs develop as females and unfertilized (haploid) eggs develop as males in uninfected hosts) and most examples of microbial-mediated parthenogenesis are in insects of the order Hymenoptera, especially wasps.
2. Male hosts are feminized, thereby doubling the number of female offspring, the same consequence as microbial-mediated parthenogenesis (described above). The principal taxa susceptible to feminization by microorganisms are Crustacea, specifically isopods (woodlice) and amphipods, but feminization of insects (e.g., the moth *Eurema hecabe*) has also been reported.
3. Uninfected eggs are killed by a factor associated with the sperm from infected hosts, but crosses between infected males and females, and between uninfected males and infected females, are fertile. (**Table 3**) This mode of host reproductive manipulation is usually known as cytoplasmic incompatibility. By killing uninfected eggs, the frequency of infected females in the population is increased, often to very high levels or fixation, at which point the microorganism has a very small, or no, impact on the reproductive output of the host population. Although the microorganisms causing cytoplasmic incompatibility occur in the testes of the male insect, the mature sperms are uninfected.
4. Preferential killing of male hosts can result in increased fitness of the female hosts (and therefore of the vertically transmitted microorganism) where the females benefit from the death of their brothers through reduced chance of inbreeding or depressed intersib competition.

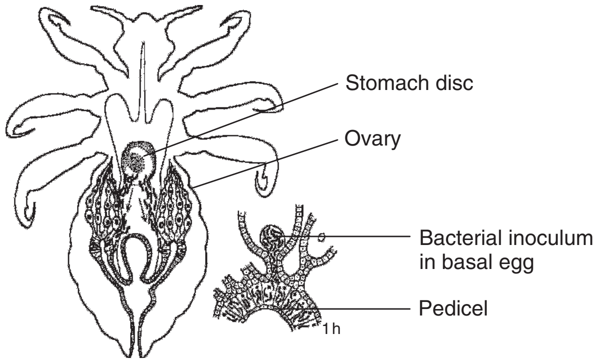

Figure 3 Vertical transmission of endosymbionts of insects. Symbiotic bacteria *Riesia* sp. in the human body louse *Pediculus humanus*. (a) Transmission of bacteria from stomach disc to ovaries of female insect. (b) Transfer of bacteria from pedicel at the base of each ovary to basal egg in one ovariole. See text for full description. Redrawn from Ries, E. (1931). Die symbiose der laüse und federlinge. *Zeitschrift fur Morphologie undÖkologie der tiere* 20: 233–367.

Most sexual eukaryotes with vertically transmitted microorganisms have an unbiased sex ratio, and this suggests that the sex determination mechanisms in most eukaryotes are difficult to distort. However, the possibility cannot be excluded that reproductive parasitism is

Table 3 Disruption of host reproduction by cytoplasmic compatibility caused by infection with the bacterium *Wolbachia*: compatible (√) and incompatible (X) crosses between hosts

	Male host	
Female host	*Uninfected*	*Infected*
Uninfected	√	X
Infected	√	√

one factor limiting the incidence of vertical transmission. Two further factors may also be important. The first is the cost of housing and nourishing symbiotic microorganisms. This may be significant at developmental stages where the microorganisms confer little or no benefit, such as during early development of the host. For example, the mycetocyte symbionts of insects occupy up to 10% of the egg volume and often proliferate rapidly after transfer to the egg, presumably utilizing the egg's nutritional reserves. The second factor limiting the incidence of vertical transmission is anatomical barriers in the host, which restrict microbial access to the gametes of the host. For example, the root symbionts of plants, for example, rhizobia and mycorrhizal fungi, are invariably horizontally transmitted, while shoot-borne microorganisms, such as bacteria in the leaf nodules of *Ardisia* species and cyanobacteria in leaflets of the water fern *Azolla*, are vertically transmitted. In animals, the gut wall is a crucial barrier to microbial colonization of the body cavity and, ultimately, the gonads; gut-borne microorganisms are generally horizontally transmitted, while microorganisms in the body cavity or internal organs (e.g., mycetocyte symbionts) are vertically transmitted. The anatomical barrier of the gut wall can, however, be bypassed by animal behavior. For example, vertical transmission of the γ-proteobacterium *Ishikawella* in the gut of stinkbugs is achieved by the maternal deposition of fecal pellets bearing these bacteria, which are consumed by the larvae immediately on hatching from the egg.

Horizontal Transmission

Chemical signaling between the microorganisms and the host plays a critical role in the establishment of symbioses with horizontally transmitted symbionts. This is particularly well understood in the relationship between legumes and nitrogen-fixing rhizobial bacteria because the symbiosis is amenable to molecular dissection, including the analysis of symbiosis formation by panels of mutants and the use of reporter genes to identify when and where individual genes are expressed as the symbiosis is established. It is now apparent that the so-called flavonoid-Nod factor-kinase signaling cascade (**Figure 4**) plays a crucial role in symbiosis formation.

The first step in this cascade is the detection of a compatible plant host by rhizobial cells in the rhizosphere. The host signal is a specific flavonoid in the root exudates that diffuses into the rhizobial cell. The rhizobium responds to this chemical signal by synthesizing a responding signal, specifically an acylated oligosaccharide called a Nod factor. The Nod factor is the signal to the plant to allow the rhizobia to colonize the root and to initiate the development of the nodule that, in due course, houses the rhizobia. It is also an important determinant of the specificity of the symbiosis. Nod factors vary in the structure of the acyl chain and number and position of acetyl and sulfate groups, and each legume species associates only with rhizobia that produce Nod factors of certain chemical structures. The molecular basis of the plant response to the rhizobial Nod factor includes a protein, specifically a serine/threonine receptor kinase with one or more LysM motifs that is predicted to bind the acetyl-glucosamine backbone of the Nod factor. The result of this molecular interaction is a signaling cascade in the plant cell, as outlined in **Figure 4**.

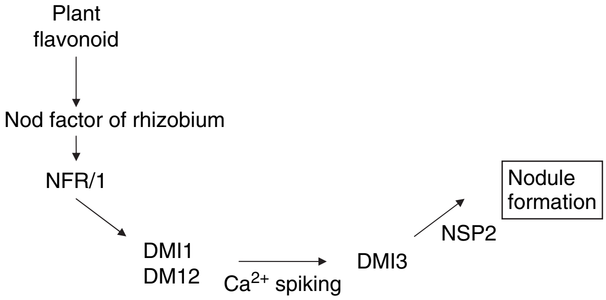

Figure 4 Signal transduction between symbiosis of alfalfa roots with rhizobia. The DMI2 protein is a receptor kinase with leucine-rich repeats, similar to plant kinases involved in defense against pathogens; DMI3 is a calcium-calmodulin kinase; and NSP2 has a GRAS domain characteristic of transcriptional regulators.

The relationship between the molecular signaling-mediated flavonoid-Nod factor and the route by which rhizobia infect the root and the development of the nodule is understood in broad outline. The initial interaction occurs at the zone of contact between the rhizobial cell and a root hair, which responds to the Nod factor by curling at the site of contact. The cellular machinery for growth in the root hair is then reorganized to create an invaginated tube, known as the infection thread, which extends through the cell toward the root cortex, with its dividing population of rhizobial cells. At this early stage in infection, the region of the cytoplasm surrounding the nucleus undergoes regular oscillations in Ca^{2+} concentration, a process known as calcium spiking. This is required for the activation of the calcium-dependent calmodulin kinase DMI3 and the transfer of the putative transcription factor NSP1/2 to the nucleus. The first sign of the developing nodule is a wave of cell division near the center of the root. It is apparent about 12 h after rhizobial contact with the root, while the infection threads and the rhizobia are extending down through the epidermal and outer cortical cells, and it results in a mass of cells known as the nodule primordium. As the infection thread extends into this region, rhizobia are endocytosed from the infection thread into cells, where they differentiate into nitrogen-fixing bacteroids. Some plant cells remain uninfected and these form the nodule meristem.

Gaining Entry into Host Cells

For intracellular microorganisms, especially horizontally transmitted taxa, access to the host cell contents is an essential feature of transmission. This topic has been studied extensively for facultatively intracellular bacterial parasites of mammalian cells. These microorganisms gain entry into the host cell by phagocytosis: some, such as *M. tuberculosis*, are phagocytosed by macrophages (specialized phagocytic cells of the immune system), but many others induce phagocytosis by cells that are not normally phagocytically active. Two broad mechanisms by which bacteria invade nonphagocytic cells have been identified: the trigger mechanism and the zipper mechanism. The bacterial proteins mediating uptake of various pathogens have been identified, and they are key virulence factors.

In the trigger mechanism adopted, for example, by *Salmonella* and *Shigella*, the effector proteins are secreted from the bacterial cell via the type III secretion system into the host cell, resulting in membrane ruffling. Actin-rich protrusions are thrown up and fold over, engulfing the bacterium. The underlying mechanisms include the activation of Rho family GTPases, such as Rac and Cdc42, and direct binding to F-actin, resulting in reorganization of the actin microfilament network. These responses of the host cell are reminiscent of their response to growth factors, suggesting that the same intracellular signaling cascades may be involved.

The zipper mechanism displayed, for example, by *Listeria monocytogenes* and *Yersinia pseudotuberculosis* to induce phagocytosis is mediated by adhesin proteins on the bacteria surface that bind to host cell surface molecules, which normally mediate adherence to other cells or the extracellular matrix. The key protein in *Y. pseudotuberculosis* is known as invasin and it binds to a subset of β_1-integrins (receptors for fibronectin), and the internalin protein of *L. monocytogenes* binds to E-cadherin. The resultant cytoskeletal rearrangements in the host cell lead to phagocytosis of the bacteria.

Intracellular symbionts also gain entry into host cells by phagocytosis, but the underlying mechanisms have not been studied extensively. Some authorities predict that internalization is mediated by specific surface molecules of the symbiont, analogous to the virulence factors of pathogens. However, studies of the uptake of symbiotic *Chlorella* by hydra suggest that the relatively nonspecific trait of surface charge may be the key discriminant of an acceptable symbiont.

Persistence of Associations

Host Controls Over Microbial Infections

Endosymbioses persist, meaning that the population of microorganisms is retained within the host for extended periods, potentially for the full lifespan of the host and, in vertically transmitted associations, through multiple host generations. Furthermore, the density and proliferation rate of the microorganisms are tightly regulated such that the microbial population increases in parallel with the host, neither overgrowing nor being diluted out by host growth. Generally, this requires suppression of microbial growth rates. For example, the doubling time of the dinoflagellate alga *Symbiodinium* is <24 h in culture and 50–60 days in symbiosis with corals; the symbiotic bacteria *Buchnera* in aphids with a population doubling time of *c.* 3 days are allied to enteric bacteria with a capacity to divide every 20 min.

The endosymbiotic microorganisms may be controlled by suppression of growth rates, expulsion from the host, and by lysis, and the relative importance of these different processes varies among associations. Most algal associations in hydra, corals, and related aquatic invertebrates are regulated primarily by controls over algal proliferation, although up to 5% of the algal population in some coral hosts may be expelled from the association per day. The algal density in these symbioses is increased in media with high concentrations of ammonia or other inorganic nutrients, suggesting that these symbionts may be nutrient-limited and that the host control over nutrient supply to the symbionts may be overwhelmed by high levels of exogenous nutrients. Expulsion plays a central role in the regulation of the bioluminescent bacteria *Vibrio fischeri* in the bobtail squid *Euprymna scolopes*, with up to 90% of the bacterial population in the squid light organ expelled daily followed by a rapid proliferation of the remaining bacterial population. Lysis of endosymbionts is developmentally controlled in many symbioses. For example, cells bearing the bacteria *Buchnera* in aphids lyse in mid-reproductive insects, releasing the bacterial cells into the hemolymph (blood), where they are destroyed.

The abundance of microbial symbionts is also influenced strongly by the scale of the benefit they confer on the host. This has been demonstrated experimentally for the symbiosis of *Bradyrhizobium* with soybean plants. When the bacteria are prevented from fixing nitrogen by replacing air with the N_2-free atmosphere of argon and oxygen, the numbers of rhizobia are markedly reduced; this effect is obtained whether the experiment was conducted at the scale of the whole root, part of the root system, or even the individual root nodule (**Figure 5(a)**). Monitoring of the oxygen relations revealed reduced oxygen tensions in the central infected zone of the nodule, where the rhizobia are located, and depressed oxygen permeability of the outer nodule tissues (**Figure 5(b)**). These results suggest that legume plants respond to rhizobia that fail to fix nitrogen by decreasing the oxygen supply to the rhizobia.

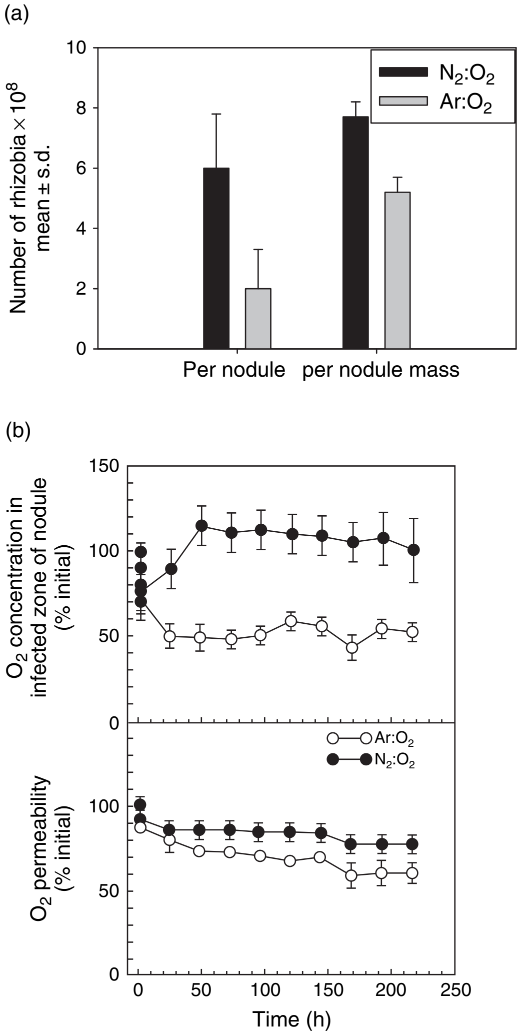

Figure 5 Impact of inhibiting nitrogen fixation by *Bradyrhizobium* symbionts in soybean plants by exposure to nitrogen-free air (Ar:O$_2$, with nodules in N$_2$:O$_2$ air as controls). (a) Number of rhizobia in nodules; (b) oxygen relations in nodules. Reproduced from Kiers *et al*. (2003) Host sanctions and the legume-rhizobium mutualism. *Nature* 425: 78–81.

The host can also control the life history traits of their symbionts, generally suppressing motile or sexual forms. For example, the fungal symbionts of leaf-cutting ants are maintained in a permanently asexual condition, presumably by secretions from the ants; sexual fruiting bodies are produced only in nests abandoned by the ants. The persistence of the obligately anaerobic protists in wood-eating cockroaches *Cryptocercus* is linked to the molt cycle of the insect. The protists are restricted to the anoxic hindgut of their insect host, where they degrade ingested cellulose. However, they are expelled from the insect at each insect molt. In the hours prior to expulsion, and in response to elevated titers of the insect ecdysteroid (molting) hormones, the symbionts develop into oxygen-resistant cysts, enabling them to survive in the external environment until such time as they are ingested by the insect and return to a metabolically active state in the insect gut.

Endosymbionts are generally restricted to specific locations in their host, such as particular organs or cell types that, in some associations, have the sole function to house and maintain the microorganisms. Examples include the root nodules of leguminous plants, the light organs of various fish and squid housing luminescent bacteria, and the bacteriocytes of diverse insects. A particularly vivid example of spatial control is provided by stratified lichens, that is, lichens in which the photosynthetic symbionts are restricted to a specific zone of the thallus (body) of the lichen. Within this layer, light capture by the symbionts is optimized by minimizing shading of one symbiont cell by another. For example, the algal symbiont *Trebouxia* in the lichen *Parmelia borreri* is maintained in regular rows, controlled by hyphae of the fungi. Each *Trebouxia* is contacted by a single fungal haustorium. When the algal cell divides to produce four daughter cells, the fungal haustorium branches fourfold and lengthens, thereby separating the four daughter cells and maintaining regular spacing between the algal cells.

Persistence of Intracellular Infections

Understanding of the processes by which microorganisms suppress or evade the defenses of their hosts is fragmentary for most systems apart from the intracellular parasites. These microorganisms manipulate the endosomal system in a diversity of ways that is not linked to the mode of entry into the cell (**Table 4**). These are considered in turn.

A particularly widespread strategy is to modify the endosomal compartment, thereby preventing lysosomal fusion. This is achieved in a variety of ways. *M. tuberculosis* arrests endosomal maturation at the 'early endosome' stage, as illustrated by the presence of the protein Rab5 (a marker for early endosomes) and the absence of Rab7 (late endosome marker) in the vacuolar membrane, while *Salmonella* species allow maturation of the endosome to the 'late endosome' (i.e., an acidic compartment with Rab7), a stage prior to lysosomal fusion, and the

Table 4 Intracellular habitats of parasitic microorganisms

Habitat	*Microorganisms*
Endosome	*Mycobacterium* species *Salmonella typhimurium*
Phagolysosome	*Coxiella burnetii*
Cytoplasm	*Listeria monocytogenes* *Shigella* species *Rickettsia* species *Trypanosoma cruzii*

compartment bearing *Chlamydia trachomatis* has features characteristic of the exocytic pathway. The apicomplexan parasite *Toxoplasma gondii* is also borne within a membrane-bound compartment, but it is generally considered to be isolated from the host cell endosomal pathway because the vacuole is constructed from *T. gondii*-derived lipids and the membrane proteins are predominantly derived from the parasite and not the host cell. However, recent data indicate that this parasite actively recruits host microtubules that act as a conduit for the delivery of membrane-bound vesicles from the endosomal system.

Some intracellular microorganisms escape from the phagosome into the cytoplasm, a trait mediated by specific microbial proteins. *L. monocytogenes* secretes a hemolysin protein known as listerolysin O and a phospholipase C, which disrupt the membrane, releasing the bacterium into the cytoplasm. The hemolysin has appreciable sequence similarity to hemolysins of other bacteria but, unusually, includes a domain with the eukaryotic PEST sequence, which triggers targeting to the proteasome and degradation. As a consequence of this domain, listerolysin O released by bacterial cells into the cytoplasm is destroyed (thereby avoiding damage to the cell membrane), while listerolysin O molecules released from bacterial cells in the phagosome are isolated from the proteasome and so remain active. The implication is that, by acquiring the PEST domain, the activation of listerolysin O is regulated according to the location of the bacterial cell. The activity of listerolysin O in the phagosome is promoted by low pH in this compartment.

Trypanosoma cruzii also resides only briefly within a host membrane after uptake. When this parasite contacts the surface of a susceptible cell, it induces local increase in cytoplasmic Ca^{2+} levels in the host cell, leading to the recruitment of lysosomes to the surface and engulfment of the parasite by membrane predominantly of lysosomal origin. The trypanosome then secretes, first, a *trans*-sialidase that mediates the transfer of sialic acid residues from host glycoproteins to the trypanosomal surface glycoproteins as a molecular ‘disguise’ and, second, a pore-forming toxin that disrupts the vacuolar membrane, releasing the parasite.

Limited data suggest that some intracellular symbionts may prevent lysosomal fusion by arresting the maturation of the phagosome. For example, the symbiosomal membrane bounding the dinoflagellate alga in corals and related marine animals includes Rab5, but not Rab7. The membrane bounding symbionts is commonly described as a symbiosomal membrane. Its composition in legume nodules bearing rhizobia has been shown to differ from the cell membrane, with a high lipid content and containing proteins uniquely expressed in the nodule (e.g., NOD26, a dicarboxylate transporter that supplies malate, an important carbon source, to the rhizobia symbionts).

Breakdown of Symbioses

Generally, endosymbioses are robust to fluctuations in abiotic conditions, but some are susceptible to particular environmental circumstances. The most intensively studied instance of symbiosis collapse is provided by coral bleaching, a phenomenon of global significance, contributing to the deteriorating condition of the world’s coral reefs. Formally, bleaching refers to the loss of color from aquatic hosts (animals and protists) with symbiotic algae. Virtually all research has concerned the symbioses of corals and other marine animals (sea anemones, tridacnid clams, etc.) with the dinoflagellate alga *Symbiodinium*, and these associations appear to be more prone to bleaching than symbioses involving other algae (e.g., diatoms in some foraminiferans, *Chlorella* in freshwater hosts). Bleaching of symbioses with *Symbiodinium* encompasses at least two syndromes. The first is the loss of *Symbiodinium* cells, commonly by exocytosis from endodermal cells, although sloughing off and loss of endodermal cells and their complement of *Symbiodinium* is also involved in some systems. Corals that appear bleached by eye may have lost from 50–70% to essentially the total algal population. The second mode of bleaching is the transformation of algal pigments to colorless products. This is particularly likely to occur in response to rapid increases in irradiance and involves little or no reduction in the density of *Symbiodinium* cells.

There is widespread agreement that the most important trigger for coral bleaching is elevated temperature, often associated with high irradiance, but other triggers, for example, low salinity, metal pollutants, low temperature, darkness, and certain bacteria may be locally important or act in combination with elevated temperature. To date, most research on the mechanisms of bleaching has focused on the primary lesion caused by elevated temperature and irradiance. This lesion is in the *Symbiodinium*, specifically the photosystem II, which mediates the stripping of electrons from water to release molecular oxygen. One scenario is that excess excitation energy arising from temperature/irradiance-induced inhibition of PSII generates reactive oxygen species (ROS), such as superoxides and oxygen radicals. ROS damage plastid thylakoids and other cell components. The host partner in bleaching symbioses also has elevated ROS and detoxifying enzymes, but it is unclear whether this is a defensive response (analogous to the oxidative burst of pathogen-challenged macrophages) to damaged algal cells or evidence that the host is also under oxidative stress. A high priority for future research is to establish how the host cells respond to this impairment of symbiont function, resulting in the collapse of the symbiosis.

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Enteropathogenic Infections

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Defining Statement
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Infections Caused by Bacterial Colonization through Attachment
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Glossary

bacteriophage Virus that infects a bacterial host.

colitis Inflammation of colon (large bowel).

cytotoxin Bacterial toxins that cause cell damage.

enterocytes Intestinal epithelial cells.

enterotoxin Bacterial toxins that induce fluid secretion from intestinal cells.

fimbriae (pili) Surface adhesins that mediate bacterial adherence to host cells.

flagellae Surface structures that provide bacteria with motility and the ability to move toward nutrients and away from toxic materials (chemotaxis).

pathogenicity island Cluster of virulence genes that is foreign and absent in the nonpathogenic strains of the same species.

quorum sensing A mechanism by which bacteria regulate their gene expression in response to cell density.

superantigen Antigens that bind to lymphocytes in a nonspecific manner and activate them.

type III secretion system A common system found in many Gram-negative bacterial pathogens as well as in symbionts of plant and animals. It consists of a syringe-like apparatus that spans the bacterial membranes and inserts into the host cell membrane and delivers secreted proteins into the host cells.

type IV fimbriae Special fimbriae with different structural subunits and different morphology from common fimbriae. These fimbriae are produced by several Gram-negative pathogens.

Abbreviations

A/E	attaching and effacing
AAF	aggregative adherence fimbriae
ail	attachment-invasion locus
Bfp	bundle-forming pili
CDT	cytolethal distending toxin
CFAs	colonization factor antigens
CPE	*Clostridium perfringens* enterotoxin
CSs	coli surface antigens
CT	cholera toxin
EAEC	enteroaggregative *E. coli*
EAST1	EAEC heat-stable enterotoxin
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
Esp	*E. coli* secreted proteins
ETEC	Enterotoxigenic *E. coli*
FAS	fluorescent actin staining
HUS	hemolytic uremic syndrome
InlA	internalin A
InlB	internalin B
Inv	invasin
LEE	locus for enterocyte effacement
LLO	listeriolysin O
LOS	lipooligosaccharide
Lpf	long polar fimbriae
LT	heat-labile enterotoxin
NAP1	American pulsed-field type 1
Nod1	nucleotide-binding oligomerization domain 1
N-WASP	neuronal Wiskott–Aldrich syndrome protein
PAI	pathogenicity islands
Pet	plasmid-encoded toxin
PMN	polymorphonuclear cells
SCV	*Salmonella*-containing vacuole

ShET1	*Shigella* enterotoxin 1	**TCP**	toxin coregulated pilus
ShET2	*Shigella* enterotoxin 2	**Tir**	translocated intimin receptor
SPI-1	*Salmonella* PAI 1	**TTSS**	type III secretion system
ST	heat-stable enterotoxin	**YadA**	*Yersinia* adhesin A
Stx	Shiga toxin	**Zot**	Zonula occludens toxin

Defining Statement

Enteropathogenic bacterial infections, manifested by syndromes such as watery diarrhea, dysentery, and enteric fever, cause high mortality and morbidity in humans. Pathogens are transmitted to humans by person-to-person contact or by contaminated food or water. They cause disease by toxin formation, by destruction of intestinal cells, or invasion.

Introduction

Enteropathogenic infections are caused by a variety of microorganisms including bacterial pathogens. These infections produce symptoms such as diarrhea and vomiting, which involve noninflammatory or inflammatory processes. Noninflammatory infections such as cholera occur mainly in the proximal small bowel and cause symptoms mainly due to increased intestinal secretion resulting from enterotoxin production. Inflammatory infections, on the other hand, occur in the distal small bowel or colon. Symptoms are due to decreased intestinal absorption that results from the destruction of the intestinal mucosa by pathogens such as *Shigella* and *Salmonella*. Enteric infections can also lead to systemic symptoms such as those seen with typhoid fever. Although enteric infections are manifested predominantly by short-term gastrointestinal symptoms, postinfection complications can ensue. For example, enterohemorrhagic *Escherichia coli* infections can result in permanent renal dysfunction, while yersiniosis can lead to chronic arthritis (Reiter's syndrome). *Campylobacter* infection may cause the neuroparalytic Guillain–Barré syndrome and typhoid fever can turn into a life-long recurrent illness.

Infections resulting in diarrhea are largely diseases of impoverished people. They are the major cause of mortality and morbidity in children from the developing world, where malnutrition, poor personal hygiene, and inadequate sanitation are commonplace. Although the mortality rate in the endemic areas has declined over the past three decades, the incidence of diarrheal diseases has remained high, particularly in children below the age of 5 years. Repeated episodes of diarrhea in these children often leave them with the lifetime complications of malnutrition and growth retardation.

In the industrialized world, diarrheal diseases are much less frequent and are often related to food-borne outbreaks and the overuse of antibiotics. Nevertheless, there has been a significant increase in the incidence of infections with the emergence of enteropathogens such as *E. coli* 0157:H7 and a *Clostridium difficile* strain that produces a more virulent toxin, which is more common in hospitals and chronic care centers. These trends have resulted in the enactment and enforcement of new food-handling policies as well as hospital infection control policies. Furthermore, in recent years, the bioterrorism threat for misuse of bacterial products as biological weapons, such as the use of botulinum toxin for massive food poisoning, has led to the establishment of new regulations for handling of the pathogens and for public health preparedness.

Compelling information from comparative genomics, *in vitro* assays using cell cultures, and *in vivo* experiments using animal models of infections suggest that the enteropathogenic bacteria use both common and specific strategies to overcome the host defense system and successfully express infections. For example, though *Salmonella typhimurium* and *Salmonella typhi* employ a similar specialized secretion system to inject their effectors into the host cells, they use different specific factors to cause a local inflammatory infection (i.e., *S. typhimurium*) or to spread and cause infection in systemic sites (i.e., *S. typhi*).

In this article, we will stratify these bacteria based upon three main mechanisms of pathogenicity. These are the following:

1. toxin delivery without prior colonization,
2. colonization through attachment with or without overt cell damage, and
3. colonization through invasion of the epithelial cells of the small or large bowel.

The prototypic pathogens for each class will be discussed, as the coverage of all the enteropathogenic bacteria is beyond the scope of this article. A brief review of the epidemiology and clinical symptoms of the illness caused by each pathogen is presented. (**Tables 1** and **2**).

Table 1 Characteristics of the bacterial enteric infections

Infection	*Pathogen*	*Route of transmission*	*Site of infection*	*Incubation period*	*Clinical syndrome*
1. Noninflammatory	*Staphylococcus aureus*	Foods high in protein, salt, or sugar	Small intestine	2–6 h	Watery diarrhea, vomiting
	Bacillus cereus (emetic)	Foods such as fried rice	Small intestine	1–6 h	Nausea, vomiting
	B. cereus (diarrheal)	Foods such as vanilla sauce	Small intestine	8–16 h	Watery diarrhea, abdominal cramps
	Clostridium botulinum	Home-canned vegetables, fruits, and fish	Small intestine	18–36 h	Nausea, vomiting, diarrhea, paralysis
	Small intestine	8–16 h 5–6 h	Watery diarrhea Necrotic enteritis		
	Vibrio cholerae	Fecal–oral, contaminated food and water, and shellfish	Small intestine	16–72 h	'Rice water' diarrhea
	ETEC[a]	Contaminated water or food	Small intestine	16–72 h	Watery diarrhea, abdominal pain
	EPEC[b,c]	Fecal–oral and person–person contact	Small intestine	12–24 h	Vomiting, watery diarrhea, fever
	EAEC[c,d]	Fecal–oral and contaminated water and food	colon and small intestine	8–18 h	Watery and mucoid diarrhea, abdominal pain, low-grade fever
2. Inflammatory	EHEC[e]	Foods contaminated with cattle feces and person–person contact	Small intestine and colon	3–8 days	Diarrhea, abdominal pain, hemorrhagic colitis, hemolytic uremic syndrome
	C. difficile	Fecal–oral and environmental contamination with spore	Colon	Variable	Diarrhea, pseudomembranous colitis
	Shigella spp.	Fecal–oral, contaminated food and water, Person–person contact	Colon	16–48 h	Watery diarrhea, dysentery
	Salmonella (nontyphoidal)	Contaminated water and foods of animal origin such as meat, poultry, egg, and dairy products	Small intestine and colon	6–48 h	Watery diarrhea, dysentery
	Campylobacter jejuni	Person–person contact, animal–person contact, fecal–oral, contaminated water and foods such as poultry, and raw milk	Small intestine, colon	1–7 days	Watery diarrhea, dysentery
3. Systemic	*Salmonella typhi*	Person–person contact, contaminated food and water	Systemic	1–2 weeks	Typhoid fever
	Yersinia enterocolitica	Fecal–oral, contaminated water or food such as milk and raw pork	Small intestine and systemic	4–7 days	Acute diarrhea, abdominal pain, fever, mesenteric adenitis
	Listeria monocytogenes	Foods such as soft cheese, raw milk, raw vegetables, and processed meat	Small intestine and systemic	11–70 days	Nausea, vomiting, diarrhea, bacteremia, meningitis, abortion, still birth

[a] Enterotoxigenic *E. coli.*
[b] Enteropathogenic *E. coli.*
[c] EPEC and EAEC elicit inflammatory responses as well.
[d] Enteroaggregative *E. coli.*
[e] Enterohemorrhagic *E. coli.*

Table 2 Pathogenic mechanisms of enteropathogenic bacteria

Pathogenicity mechanism	*Organism*
I. Toxin delivery without prior colonization	*Staphylococcus aureus*, *Bacillus cereus* (emetic), *Clostridium botulinum*
II. Attachment and toxin elaboration	
Overt tissue damage	EAEC[a], EHEC[b], EPEC[c], *Clostridium perfringens*, *Clostridium difficile*
No overt tissue damage	*B. cereus* (diarrheal), *Vibrio cholerae*, ETEC[d]
III. Invasion	*Shigella*, *Salmonella*, *Yersinia*, *Campylobacter jejuni*, *Listeria monocytogenes*

[a] Enteroaggregative *E. coli.*
[b] Enterohemorrhagic *E. coli.*
[c] Enteropathogenic *E. coli.*
[d] Enterotoxigenic *E. coli.*

Infections Caused by Preformed Toxins in the Absence of Bacterial Colonization

These infections are usually of a food-borne origin that can occur sporadically or as an outbreak. The common feature among these infections is the short incubation time and symptoms of nausea and vomiting due to the action of toxins. The main etiological pathogens of these infections discussed below are *Staphylococcus aureus*, *Bacillus cereus* (emetic type), and *Clostridium botulinum.*

Staphylococcus aureus

Staphylococci are Gram-positive, facultatively anaerobic, nonmotile, and non-spore-forming cocci that appear in pairs, short chains, and grapelike clusters. The genus *Staphylococcus* contains many species that are divided based on the production of coagulase. *S. aureus*, a coagulase-positive species, is one of the most virulent species. This species can be found on the skin and the anterior part of nostrils of adults. *S. aureus* causes a variety of infections and is among the most common causes of food-borne infections worldwide. Food poisoning is characterized by a short incubation period of 2–6 h after ingestion of preformed enterotoxin in contaminated food, followed by vomiting and diarrhea, which usually resolves within 6–12 h. The most common contaminated foods involved in outbreaks are those high in protein, salt, or sugar content such as ham, poultry, egg salad, and dairy products.

S. aureus produces 15 enterotoxins that function both as gastrointestinal toxins and as superantigens. Enterotoxin types A, B, C, D, and E are mostly associated with food poisoning. The total dose of enterotoxin necessary for intoxication is as low as 200 ng. These toxins are all resistant to heat denaturation and therefore not inactivated by cooking. They are also resistant to inactivation by gut proteases such as pepsin and trypsin. Structurally, all enterotoxins are monomeric proteins encoded by genes that are located on the bacterial chromosome or sometimes carried by a specific bacteriophage. The mechanism of action of these enterotoxins on the enteric nervous system and production of gastrointestinal symptoms in humans is not yet fully understood. It is, however, postulated that the gastrointestinal effect of the superantigen toxin is the result of its indirect action on the central autonomic nervous system instead of its direct secretory effect on the intestine.

Bacillus cereus (Emetic Type)

B. cereus is a Gram-positive, aerobic, spore-forming, motile rod that resides in the soil environment. It is part of the gut microflora of invertebrates in both spore and vegetative forms. It spreads to the foods of plant origin, but can also be isolated from meat, eggs, and dairy products. This organism is associated with food poisoning mostly in immunocompromised individuals or patients recovering from surgery. Food poisoning in humans occurs in two different ways: the emetic (intoxication) type, characterized by nausea and vomiting with an incubation period of 1–6 h, and the diarrheal (infection) type manifested by abdominal cramps and diarrhea with an incubation period of 8–16 h. Regardless of the type of food poisoning, the food involved is already contaminated prior to cooking. Spores can survive extreme temperatures and then germinate and multiply when allowed to cool down slowly.

The emetic syndrome is caused by the emetic toxin cereulide, which is produced by multiplying bacteria in food and/or is ingested as a preformed toxin. The short incubation period is consistent with this pathogenic mechanism. Cereulide causes only vomiting when fed to *Rhesus* monkey and, unlike the diarrheal enterotoxin, does not produce fluid accumulation as in rabbit ileal loops. The toxin is resistant to heat, extreme pH, and proteolytic enzymes. It consists of a ring structure formed by three repeats of four amino acids with a molecular weight of 1.2 kDa. Cereulide binds to the receptors on the enteric nerves, but its mechanism of action is not yet known.

The emetic syndrome has been commonly associated with fried rice and occurs about 10 times more frequently than the diarrheal type in Japan. The diarrheal form, caused by complex enterotoxins produced during the vegetative growth of the pathogen in the small bowel, is the more frequent form reported in Europe and North America. Food poisoning by *B. cereus*, in both forms, is underreported since both types of illness are relatively mild and usually last for less than 24 h.

Clostridium botulinum

C. botulinum is an anaerobic, Gram-positive, spore-forming rod that is ubiquitous in soil and aquatic environments. The strains of *C. botulinum* are classified into seven types, A–G, based on the neurotoxin serotype they produce. This organism causes botulism, a severe neurological disease, in both human and animals. Human botulism is caused mainly by serotypes A, B, E, and rarely F. In humans, botulism can occur naturally in three different forms: wound infection, infant botulism, and food-borne botulism. Wound and infant botulism result from the growth of *C. botulinum* spores in deep wounds or in an infant's intestine with subsequent *in vivo* production of toxin. In healthy adult humans, the spores, if ingested, are excreted from the intestine without germination and toxin production. Another form of botulism is inhalational, which does not occur naturally, but has been used in the past as a bioweapon.

Food-borne botulism occurs by ingestion of preformed toxin from contaminated food. The incidence of this form of botulism is low because the toxin is heat labile and can be destroyed if heated at 85 °C for 5 min. The major source of this type of botulism is home-canned vegetables, fruits, and fish that have been inadequately processed and consumed without cooking. Symptoms of nausea, vomiting, and diarrhea appear 18–36 h before or simultaneously with neuro-paralytic symptoms.

All botulinum neurotoxins consist of large single-chain polypeptides with a molecular weight of 150 kDa. They associate with other nontoxic proteins and form large complexes. The mode of action of these toxins on the nervous system is to block the release of acetylcholine at the peripheral synaptic nerve endings, causing a neuromuscular blockade and resulting in muscle relaxation. Botulinum toxin is the most potent toxin known. Primate studies suggest a lethal dose of 70 mg when taken orally. Contamination of the food supply by this toxin as a weapon for bioterrorism has created a major fear in recent years in the Western world.

The intestinal symptoms of food-borne botulism are caused by the action of cytotoxins C2 and C3. The C2 toxin ADP-ribosylates G actin at Arg^{177} and prevents polymerization of G actin to F actin. The C3 toxin ADP-ribosylates the Rho proteins A, B, and C at Asp^{41} within the GTPase effector region. These functions result in the disassembly of actin filaments, which in turn leads to the loosening of intercellular tight junctions. The role of these toxins in disease is not well-known.

Infections Caused by Bacterial Colonization through Attachment

Attachment and Elaboration of Toxin without Cytoskeletal Rearrangement

Enteropathogenic bacteria can attach to enterocytes via special adhesins and without significant alteration of the cytoskeleton of microvilli. These enteropathogens, exemplified by *V. cholerae*, often produce enterotoxins that account for noninflammatory diarrhea in the small intestine via alteration of ion transport.

Vibrio cholerae

V. cholerae is a Gram-negative bacterium belonging to the family of Vibrionaceae. The bacterium was discovered by Filippo Pacini in 1854 and by Robert Koch in 1883. It resides in aquatic environment as the microflora. During epidemics, surface water becomes heavily contaminated with *V. cholerae* through the feces of infected humans. Transmission of the infection occurs by the fecal–oral route and by ingestion of contaminated water or undercooked shellfish. The resulting illness, cholera, is characterized by vomiting and acute diarrhea with typical 'rice water' stools within 16–72 h of ingestion of the pathogen. Cholera leads to rapid severe dehydration and becomes fatal in the absence of effective rehydration therapy. *V. cholerae* is sensitive to low pH of gastric acid; thus, a high inoculum dose (10^9 organisms) is required to colonize the small intestine and efficiently cause the disease.

There are more than 200 known O serogroups of *V. cholerae*, although only serogroups O1 and O139 have been associated with cholera epidemics. The non-O1, non-O139 strains cause only sporadic cases of cholera. The O1 strains are endemic in the Indian subcontinent and have caused seven cholera pandemics throughout the rest of the world since 1817. The serogroup O139 was the cause of a cholera epidemic in 1992 in Bangladesh, India, and neighboring countries. The adult population of these areas is already immune to serogroup O1 strains. Currently, two types of vaccine, inactivated and live attenuated, are used by travelers from industrialized countries traveling to endemic areas.

The key pathogenic factors responsible for the secretory diarrhea by O1 and O139 strains are the production of type IV fimbriae, called toxin coregulated pilus (TCP), and the production of a potent enterotoxin known as cholera toxin (CT). TCP and CT are both encoded by genes located on a lysogenic bacteriophage and their expression is negatively regulated by quorum sensing.

CT is a multimeric protein consisting of five B subunits that bind to GM1 gangliosides on the surface of enterocytes and facilitates the release of a single catalytic A subunit. The A subunit catalyzes the ADP-ribosylation of the GTP-binding protein, Gs_{α}. This results in activation of adenylate cyclase, production of high level of cAMP in enterocytes, and increased secretion of chloride ions and water into the gut, which accounts for the massive watery diarrhea characteristic of cholera. CT also upregulates expression and secretion of several proinflammatory and anti-inflammatory cytokines (e.g., IL-1, IL-6, and IL-10) by enterocytes.

V. cholerae also produces another bacteriophage-encoded enterotoxin called zonula occludens toxin (Zot). Zot activates the paracellular secretion pathway by affecting the structure of the intercellular tight junction, the zonula occludens.

Enterotoxigenic E. coli

E. coli was first described by the German physician Escherich in 1885. It resides within the intestine of warm-blooded animals. In human gut, *E. coli* lives naturally as the most common facultative anaerobe, although there exist many pathogenic strains of *E. coli* that cause human and animal diseases such as enteric infections.

Enterotoxigenic *E. coli* (ETEC) pathotype is the most frequent bacterial cause of diarrhea in infants and children of the developing world, especially in tropical regions, and a common cause of travelers' diarrhea in international visitors to these areas. The disease occurs following ingestion of contaminated water or food. Abdominal pain and watery diarrhea result within 16–72 h and usually resolve within 3–4 days. In travelers the disease is usually mild, but in infants from endemic areas it is typically more severe. Repeated infections with ETEC during childhood may lead to long-term malnutrition. Epidemiological studies indicate that ETEC infection is more frequent during the warm seasons in developing countries and also in tropical areas, where there is a heavy burden of the pathogen. Studies in human volunteers have shown that, similar to *V. cholerae*, the infecting dose for ETEC is also high (10^8 bacteria).

Pathogenesis of the illness caused by ETEC resembles that of cholera, in which the pathogen colonizes the small bowel by attachment and production of the enterotoxins that induce secretion of water and electrolytes. Bacteria bind to the receptors on the epithelial cells in the small bowel lumen using fimbrial or nonfimbrial adherence factors. More than 25 types of adhesins, called coli surface antigens (CSs) or colonization factor antigens (CFAs), have been identified in human ETEC strains. These adhesins are associated with specific O serogroups and include common fimbriae with rigid rods, bundle-forming flexible rods, thin fibrillas, and type IV fimbriae.

Once attached to the enterocytes, ETEC secrete two types of enterotoxin, known as heat-labile (LT) and heat-stable (ST) enterotoxins, both encoded on a plasmid. LT is homologous to CT in structure, function, and mode of action. It has a total molecular mass of 84 kDa and is composed of a single A subunit and five identical B subunits. The B subunits bind to GM1 ganglioside receptors of the plasma membrane of the enterocyte, allowing the release of the A subunit into the cytoplasm. There, the A subunit catalyzes the activation of adenylate cyclase by ADP-ribosylation of the G proteins, which results in the accumulation of cyclic AMP in the cytoplasm of the enterocyte. This results in increased secretion of chloride ions in the crypt cells and reduced absorption of sodium and chloride ions by villous cells, accumulation of electrolytes and water in the lumen and, heretofore, watery diarrhea. LT also causes diarrhea by influencing the metabolism of prostaglandins and stimulating neurotransmitters of the enteric nervous system.

ST is a small polypeptide that is homologous to the intestinal hormone, guanylin. ST activates guanylate cyclase, which results in increased intracellular levels of cyclic GMP and secretion of electrolytes and water, leading to diarrhea. ST alone is produced by approximately half of clinical strains of ETEC, whereas most of the rest produce both ST and LT.

Attachment Followed by Cytoskeletal Rearrangement

Intestinal pathogens can adhere to the epithelial cells and remain extracellular. However, the adherence can be intimate and can lead to the loss of the brush border microvilli followed by major rearrangements in the actin cytoskeleton. The representative pathogens in this category include enteropathogenic *E. coli*. These pathogens can also secrete potent enterotoxins and cytotoxins and cause noninflammatory as well as inflammatory symptoms.

Enteropathogenic E. coli

Enteropathogenic *E. coli* (EPEC) is one of the major causes of infantile diarrhea worldwide, with a high mortality rate in infants below 2 years of age in developing countries. EPEC infections are more prevalent in tropics and during warm months. The pathogen is transmitted by the fecal–oral route by person-to-person contact. Outbreaks in developed countries usually occur in day care centers. More than 10^8 organisms are required to cause infection in adult human volunteers. The symptoms of the infection are often severe and include acute watery diarrhea, malaise, vomiting, and fever.

EPEC strains do not produce enterotoxins and are not invasive. Instead, the defining feature of this pathogen is formation of attaching and effacing (A/E) lesions on the brush border of the small intestine. The lesions are caused by the intimate contact of the bacteria with the apical enterocyte membrane followed by their effacement. Bacteria induce formation of actin-rich cup-like pedestals at the site of bacterial contact on the epithelial cell surface. These lesions have been demonstrated both in intestinal tissue biopsy of infants with diarrhea and in cell culture models by using the fluorescent actin staining (FAS) technique. The genes involved in the formation of A/E lesions are located on a 35 kb pathogenicity island called the locus for enterocyte effacement (LEE). LEE is located on a chromosome and contains more than 40 genes responsible for the production of components of the type III secretory system, a series of effector proteins called *E. coli*-secreted proteins (Esp), the 94 kDa outer membrane adhesin intimin, and the 90 kDa protein translocated intimin receptor (Tir).

The initial adherence of EPEC to the enterocytes is mediated by the plasmid-encoded type IV pili, also called bundle-forming pili (Bfp). Bfp are characterized by a rope-like morphology. *In vitro*, by using tissue culture assays, Bfp were shown to mediate the formation of tight microcolonies of 5–200 individual bacteria with a distinctive pattern of adherence to the cells called 'localized adherence'. Fimbriae, known as the surface-associated filament (EspA filaments), and flagella also contribute to the adherence process. The role of Bfp in virulence of EPEC has been shown in volunteers who developed a milder diarrhea when they ingested the Bfp mutants.

Meanwhile, localized adherence is followed by the injection of Tir into the intestinal epithelial cells through the type III secretion system (TTSS). Tir acts as the receptor for intimin and, once bound, it causes accumulation of cytoskeletal proteins such as actin, loss of microvilli, and the formation of the cup-like pedestal for the pathogen to sit on. This intimate contact leads to the secretion of Esp proteins into the cytosol of enterocytes via type III secretion apparatus. EspA forms a tunnel between the bacteria and the host cells to allow other effector proteins to translocate into the host cytoplasm. One of these proteins, EspF, modifies other host proteins and contributes to the alteration of the permeability of tight junctions. In addition, proinflammatory cytokines such as IL-8 are upregulated during infection, perhaps by flagella, which can lead to an inflammatory process that exacerbates intestinal damage. Therefore, A/E lesions may contribute to the pathogenesis of diarrhea by altering the absorptive capacity of the intestinal mucosa, which results in electrolyte and water loss, and subsequent diarrhea.

Enterohemorrhagic E. coli

Enterohemorrhagic *E. coli* (EHEC) is an emerging pathogen in the highly industrialized countries such as Japan, North America, and Europe, but causes fewer outbreaks in the developing countries. Infection occurs in both adults and children and starts with abdominal pain, vomiting, and watery diarrhea. Fever is uncommon. Watery diarrhea can progress to hemorrhagic colitis within 3–5 days. Symptoms of bloody diarrhea may then last for up to a week or more. The severe complications of hemolytic uremic syndrome (HUS) may also occur, which can lead to long-term renal damage and significant mortality rate.

The intestinal tract of cattle is the natural reservoir of EHEC and the bacteria can spread from animal feces to water and food. Transmission usually occurs through contaminated ground beef, raw milk, and vegetables, direct contact with farm animals, or by person-to-person contact. Less than 100 organisms can lead to infection. Of the 200 recognized EHEC serotypes, O157:H7 is the most common serotype in food-borne outbreaks. It was first detected as a human pathogen in 1982 after an outbreak associated with undercooked hamburger at a fast-food restaurant chain.

The main pathogenic feature of EHEC is the production of cytotoxins called Shiga toxins (or verotoxins): Stx1 and Stx2. These toxins are closely related to the Shiga toxin of *Shigella dysenteriae* (see '*Shigella* spp.'). Stx2 is the more virulent toxin and is produced by almost all O157:H7 strains. The Shiga toxins have the A1B5 structure similar to the LT toxins of ETEC and CT of *V. cholerae* (see preceding). Genes encoding Stx proteins are located on a temperate bacteriophage that has integrated in the EHEC chromosome. The mode of action of the toxin is characterized by the inhibition of protein synthesis in colon epithelial cells, resulting in cell death. The mucosal damage in the colon is similar to that seen with *C. difficile* infections. The toxin is then absorbed from the gut into the circulation where it further damages vascular endothelial cells in organs such as colon and kidney, resulting in worsening hemorrhagic colitis (similar to ischemic colitis) as well as HUS.

Infection with EHEC also results in histopathologic alterations of the intestinal mucosa and formation of typical A/E lesions similar to EPEC infections. These lesions, however, are different in composition and may develop in the colon or the distal small bowel. EHEC, like EPEC, uses the adhesin intimin (for intimate attachment), but unlike EPEC it does not produce Bfp (see preceding). EHEC also harbors a large virulence plasmid called pO157 that encodes a hemolysin called enterohemolysin (HlyA), an autotransported serine protease EspP (involved in the cleavage of human coagulation factor V), and a large protein called ToxB with structural similarity to *C. difficile* toxin. EHEC also produces the enterotoxin EAEC heat-stable enterotoxin (EAST1) that is a heat-stable protein similar to the heat-stable toxin of ETEC (see preceding).

Enteroaggregative E. coli

Enteroaggregative *E. coli* (EAEC) was first described in 1987 and is now recognized as an emerging pathogen that causes pediatric diarrhea. It is the cause of persistent diarrhea and malnutrition largely in children below the age of 2 in developing and tropical countries. It is also the cause of acute diarrhea in travelers to developing areas, in HIV-infected individuals, and in children and adults of both developing and developed countries. In addition, EAEC has been associated with large diarrheal outbreaks in Japan, Europe, and India. EAEC is transmitted by the fecal–oral route and by consumption of contaminated water and food. An inoculum dose of 10^{10} organisms was required for development of diarrhea in a volunteer study. The symptoms of disease appear within 8–18 h of ingestion of the pathogen and include watery diarrhea with or without blood and mucus, abdominal pain, nausea, and low-grade fever. In malnourished children, diarrhea can become chronic and persistent. In addition, infection with EAEC in children may result in long-term complications of malnutrition and growth retardation.

The pathogenesis of EAEC is complex, and EAEC strains are heterogeneous in terms of their virulence. Animal and cell culture studies indicate that EAEC adheres to the mucosa in both the small and the large bowel and then forms aggregates with a 'stacked brick' pattern. The adherence and aggregative phenotype is mediated by adhesins such as aggregative adherence fimbriae (AAF). AAF is encoded on a large plasmid and regulated by the AggR regulatory protein. In addition, an antiaggregative protein, called dispersin (aap), and a mucinase, called Pic, cause bacterial dispersal across the intestinal mucosa. Adherence and aggregation are followed by production of a thick layer of mucus within which bacteria adhere to each other and to the epithelium in a manner similar to biofilm. This biofilm formation accounts for the production of mucoid stool, malnutrition, and perhaps persistent colonization with subsequent diarrhea and worsening malnutrition in children with previous malnutrition. Following adherence, EAEC secretes both enterotoxins and cytotoxins. The toxins induce mild but significant cytotoxic effect on the tips and sides of the intestinal villi and epithelial cells, as seen in both animal and human studies. The damage to intestinal mucosa is different from the lesion caused by EPEC. The toxins also induce mild inflammatory response and intestinal secretion. Predominant toxins include the plasmid-encoded EAST1, *Shigella* enterotoxin 1 (ShET1), and plasmid-encoded toxin (Pet). EAST1 is encoded by astA and is a heat-stable protein similar to the heat-stable toxin of ETEC (see preceding). ShET1 is an enterotoxin similar to that of *Shigella* (see '*Shigella* spp.'). Pet is a member of a serine protease autotransporter family of bacterial toxins. It acts intracellularly and cleaves the cytoskeletal proteins spectrin and fodrin. This, in turn, leads to the loss of actin stress fibers and rounding of cells in culture. Pet also elicits enterotoxic effects and causes fluid secretion and diarrhea. In addition, the EAEC flagella induces the release of the proinflammatory cytokine IL-8, which facilitates intestinal fluid secretion. EAEC also possesses pathogenicity islands (PAI) including *Shigella* she PAI containing the enterotoxin and mucinase gene and *Yersinia* PAI containing the yersiniabactin siderophore gene.

Clostridium perfringens

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming, nonmotile rod. It is ubiquitous in nature and mainly present in soil and in the intestine of humans and warm-blooded animals. The species is divided into five types, A–E, based on the production of four major toxins. Of these types, A and C are responsible for two very different food-borne infections. Type A results in a watery diarrheal illness, while type C generally produces a more severe necrotic enteritis.

C. perfringens type A is a common cause of food poisoning outbreaks in industrialized nations, particularly in institutions where large amounts of food are cooked in advance, but not reheated adequately prior to serving. Like other toxinogenic strains, type A strains are not able to produce several essential amino acids. For this reason, they tend to associate with foods rich in protein such as meat and poultry where they can grow at temperatures between 15 °C and 50 °C, with doubling times as short as 8 min. Initial cooking kills the vegetative cells but not the spores, which will eventually germinate after cooking. Once ingested at a high inoculation dose ($\sim 10^7$ organisms), *C. perfringens* enterotoxin (CPE) is efficiently produced in the small intestine. Abdominal pain and mild watery diarrhea appear within 8–16 h and can resolve after 24 h. CPE is also responsible for the antibiotic-associated diarrheal symptoms caused by *C. perfringens* type A. As in cases of *C. difficile* infection, the diarrhea usually lasts longer and is typically more severe, being characterized by the passage of bloody mucus.

CPE is a heat- and pH-labile 35 kDa single polypeptide chain with a two-domain structure. The C-terminal domain binds to Claudin-3 and Claudin-4, the proteins located in the epithelial tight junctions, and the N-terminal domain is involved in insertion and cytotoxicity. By interaction with several membrane proteins, a pore-forming complex that increases apical membrane permeability to ions in human intestinal Caco-2 epithelial cells is formed. The cytotoxic activity of CPE has also been demonstrated in HeLa cells and animal models.

On the other hand, *C. perfringens* type C causes necrotic enteritis known as 'pig-bel', a severe necrotizing disease of the small intestine. In Papua New Guinea, outbreaks of 'pig-bel' are related to the consumption of large amount of undercooked pork during the long traditional feasts. The symptoms of 'pig-bel' appear within 5–6 h of ingestion of the pathogen, characterized by severe abdominal cramps, vomiting, bloody diarrhea, and eventually death due to intestinal perforation. The disease is mainly due to the production of a cytotoxin called b-toxin, produced by the vegetative cells. This toxin is degraded by proteolytic enzymes such as trypsin in the normal human intestine. However, the lack of proteolytic enzymes in the diet due to malnutrition and consumption of foods containing trypsin inhibitors, such as sweet potatoes, is a precipitating factor in the development of the disease.

Clostridium difficile

C. difficile is an anaerobic, spore-forming, Gram-positive rod that exists naturally in the environment. It is the most commonly identified cause of diarrhea in hospitals worldwide. The incidence of *C. difficile* infections has increased in developed nations since the 1980s as a result of increased use of antibiotics. The antibiotics alter the colonic microflora and allow colonization of the intestinal tract by *C. difficile*. The spores, which are resistant to many disinfectants used in the hospitals, are able to survive the acidic environment of the stomach and germinate in the colon, subsequently producing toxins. The clinical spectrum associated with *C. difficile* infection can range from mild diarrhea to severe pseudomembranous colitis. Recurrence of diarrhea is also frequently observed.

C. difficile produces two toxins, toxin A and B, which are responsible for the pathophysiology of diarrhea and colitis. The expression of these toxins is coregulated. In addition, toxins A and B are among the largest monomeric toxins known, with a molecular weight of 308 and 207 kDa, respectively. Both the toxins exert general cytotoxic activities although toxin A has been traditionally known as an enterotoxin. Both the toxins have glucosyltransferase activities that catalyze transfer of glucose to small GTP-binding proteins belonging to the Rho family. This modification results in the disruption of the actin cytoskeleton and leads to rounding of the cells. These toxins also cause alterations of the intestinal barrier function most likely through a proteinase kinase C signaling pathway. In addition, toxin A binds to colonocytes and enters the mitochondria prior to the process of Rho glycosylation, leading to early release of proinflammatory cytokines such as IL-8. This accounts for the severe inflammation often seen with pseudomembranous colitis.

Another toxin, called binary toxin, is produced only by some strains. Binary toxin causes fluid secretion with no epithelial damage in the rabbit ileal loop, but its role in disease development is currently unknown. Other factors contributing to the pathogenicity of *C. difficile* include fimbriae, the capsule, and hydrolytic enzymes. Moreover, mutations in one of the regulatory genes of toxin A and B has been shown to result in constitutive production of the toxins by the pathogen. Such a strain is then defined as hypervirulent and is associated with a high rate of *C. difficile*-associated complications such as toxic megacolon (which may result in colectomy) or even death. Thus the hypervirulent strain, referred to as PCR ribotype 027 or American pulsed-field type 1 (NAP1), is a new emerging strain that is resistant to fluoroquinolone antibiotics and was responsible for an epidemic in Quebec, Canada, in 2003. In addition, this strain has caused other outbreaks in the United States and Europe since then (**Table 3**).

Infections Caused by Bacterial Colonization through Invasion

Enteroinvasive pathogens invade the mucosa of the distal ileum or colon following adherence to the intestinal epithelia and this usually results in an inflammatory

Table 3 Predominant virulence factors of enteropathogenic bacteria

Pathogen	*Virulence factor*
Bacillus Cereus	Cereulide (enterotoxin)
Campylobacter jejuni	Cytolethal distending toxin (CDT), flagella
Clostridium botulinum	Botulinum toxin (enterotoxin), C2 and C3 (cytotoxins)
Clostridium difficile	Toxin A and B (cytotoxin)
Clostridium perfringens	*Clostridium perfringens* enterotoxin (CPE), b-toxin (cytotoxin)
Enteroaggregative *E. coli* (EAEC)	Aggregative adherence fimbriae (AAF), dispersin, mucinase, EAST1[a], ShET1[b], plasmid-encoded toxin (Pet)
Enterohemorrhagic *E. coli* (EHEC)	Stx1 and Stx2[c] (cytotoxins), intimin
Enteropathogenic *E. coli* (EPEC)	Intimin, bundle forming pili (Bfp)
Enterotoxigenic *E. coli* (ETEC)	Heat-labile (LT) and heat-stable (ST) enterotoxins, colonization factor antigens (CFAs)
Listeria monocytogenes	Internalin A and B, listeriolysin O, Act-A
Salmonella (nontyphoidal)	*Salmonella* enterotoxin, Sips[d], TTSS[e]
Salmonella typhi	Vi capsule, PhoP/PhoQ system, Sips, TTSS
Shigella	Stx[c] (cytotoxin), Ipas[f], TTSS, IcsA/VirG
Staphylococcus aureus	Enterotoxins type A, B, C, D, and E
Vibrio Cholerae	CT[g] (enterotoxin), toxin coregulated pilus(TCP), zonula occludens toxin (Zot)
Yersinia enterocolitica	Invasin (Inv), Yst (enterotoxin), Yops[h], TTSS

[a] EAEC heat-stable enterotoxin.
[b] *Shigella* enterotoxin 1 (ShET1).
[c] Shiga toxins 1 and 2.
[d] *Salmonella* invasion proteins.
[e] Type III secretion system.
[f] Invasin plasmid antigens.
[g] Cholera toxin.
[h] *Yersinia* outer membrane proteins.

type of diarrhea. Some of these pathogens may remain localized and only invade the epithelium, thus causing serious inflammatory destruction of the colonic mucosa (e.g., *Shigella*). Other pathogens may further infect mesenteric lymph nodes draining the intestine (e.g., *Yersinia*) or spread beyond to the extraintestinal sites and result in systemic infection (e.g., *S. typhi*). The best-characterized pathogens in each category are discussed in the following sections.

Local Invasion

Shigella *spp.*

Shigella spp. are Gram-negative, nonmotile, nonencapsulated, facultatively anaerobic bacilli and were first described by Shiga at the end of the nineteenth century. They are genetically closely related to *E. coli.* Based on their biochemical and serological characteristics, they are divided into four different species: *S. dysenteriae*, *S. flexneri*, *S. bodyii*, and *S. sonnei*. *S. dysenteriae* serotype 1, the Shiga bacillus, is responsible for most recorded epidemics in the developing countries. *S. bodyii* exists mostly in the Indian subcontinent while *S. flexneri* and *S. sonnei* are responsible for most of the endemic diseases. *S. sonnei* is prevalent in Europe and the United States, whereas, *S. flexneri* is endemic in developing countries and characteristically produces higher mortality rates compared to the other species. *Shigella* is the etiologic agent of shigellosis or bacillary dysentery, accounting for approximately 5% of all the episodes of diarrhea. Mortality due to shigellosis is high in developing countries, since it largely affects children between 1 and 5 years of age. In recent years, there has been a decline in mortality rate, although the incidence of the disease has remained high in endemic areas.

The symptoms of shigellosis occur within 16–48 h of ingestion of the pathogen and range from self-limiting watery diarrhea to the classic triad of fever, abdominal cramps, and bloody diarrhea. The infectious dose for the organism is as low as 10–100 organisms in adult volunteers. Transmission usually occurs by person-to-person contact or through contaminated food and water. Infection occurs primarily as the result of poor hygiene and inappropriate sanitation.

Shigella is the paradigm of enteropathogenic bacterial invasion that results in an inflammatory infection. Once the bacteria reach the colon, they invade the mucosa by penetrating, multiplying, and spreading between the colonocytes. Genes required for these key steps of invasiveness are located on a 221 kb virulence plasmid, called pWR100. These genes are clustered on PAI. The main PAI is a 30 kb fragment that carries genes encoding the components of a TTSS) called Mxi-Spa, the secreted effector proteins called Ipas, and the chaperones for these proteins called Ipgs. Like other TTSSs, the Mxi-Spa TTSS has a syringe-like structure composed of three components: a cytoplasmic bulb, a disk-like structure that spans the inner and outer membrane, and a 60 μm needle-like component that extends outside the outer membrane (**Figure 1**).

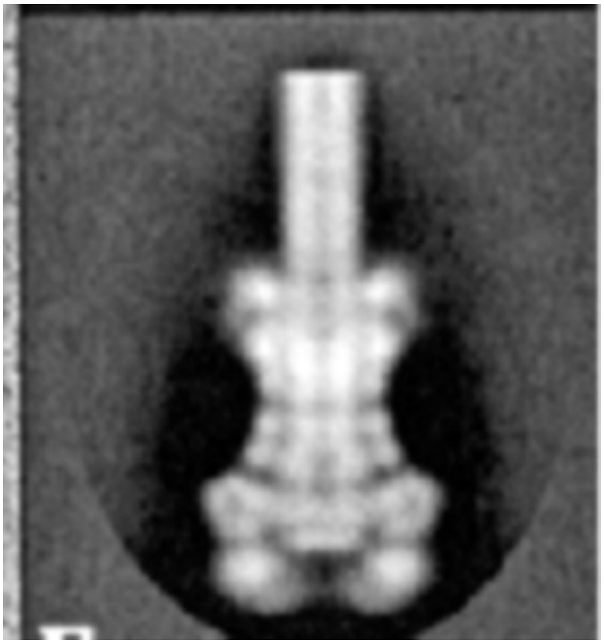

Figure 1 Computer reconstitution of a *Shigella* type III secretion system (TTSS), based on addition of a large set of electron microscopy pictures. TTSS from other Gram-negative enteric pathogens (i.e., *Salmonella*, *Yersinia*) are structurally similar.

Shigella strains do not invade through the apical membrane of the epithelial cells. Experimental infections in the rabbit ligated ileal loop have shown that once inside the intestine, *Shigella* selectively translocate across the intestinal epithelium via the endocytic M cells. Following translocation, the bacteria are phagocytosed by resident macrophages. Subsequently, they destroy the macrophages by IpaB-mediated apoptosis, thereby gaining access to the basolateral membrane of the epithelial cells, which they can then efficiently enter. Upon contact with the host cell, the TTSS becomes activated, leading to the secretion of IpaB and IpaC and their insertion into the plasma membrane of the host cell. The IpaB–IpaC complex forms a pore that can facilitate the insertion of the needle component into the target cell membrane. The pore also allows passage of effector proteins such as IpaA and IpgD directly from the bacterial cytoplasm into the target cell. IpaC also induces actin nucleation/polymerization at the early stage of entry by activating the cytoskeletal proteins, Cdc42, Rho, and Rac. IpaA activates vinculin, which causes the formation of the actin cup structure beneath the bacterium. IpgD facilitates extension of actin filaments. Therefore, *Shigella* employs a 'triggering' mechanism that induces cytoskeletal rearrangements, with the formation of ruffles and the engulfment

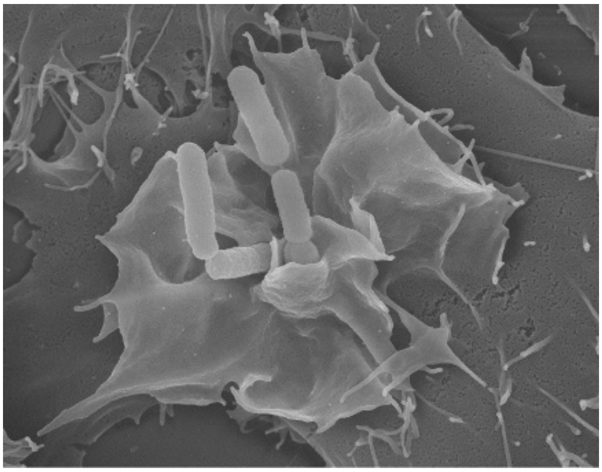

Figure 2 Entry of *Shigella flexneri* into an epithelial HeLa cell. Note massive membrane rearrangements that form a macropinocytic pocket in response to the cytoskeletal changes induced by the type III secreted effectors.

of the bacteria within macropinocytic vacuoles (**Figure 2**). *Shigella* then lyses the vacuoles by using the IpaB invasion and escapes into the cytoplasm, where it multiplies. Within the cytoplasm, *Shigella*'s outer membrane protein, IcsA/VirG, binds to the neuronal Wiskott–Aldrich syndrome protein (N-WASP) and causes nucleation/polymerization of actin and formation of an F-actin comet at one pole of the bacterium. This enhances the ability of the bacterium to move inside epithelial cells. The motile *Shigella* can then recruit the components of the cell intermediate junction such as cadherins to form membrane protrusions that are endocytosed by the adjacent cell. Following internalization of the protrusion, the two membranes are lysed by the IpaB/IpaC pore complex, allowing cell-to-cell spread of the bacterial pathogen. This cell-to-cell spread can be measured *in vitro* by the formation of plaques on a confluent cell monolayer (plaque assay) and *in vivo* by formation of keratoconjunctivitis in guinea pigs (Sereny test).

As expected, invasion by *Shigella* causes severe local inflammation. Apoptosis of macrophages leads to release of proinflammatory cytokines such as IL-1 and IL-18. In addition, once intracellular, a small motif of peptidoglycan of the bacterial cell wall binds to the nucleotide-binding oligomerization domain 1 (Nod1) protein of the epithelial cell and induces secretion of the proinflammatory cytokine IL-8. These events lead to the infiltration of the polymorphonuclear cells (PMN) through the epithelial cell tight junctions, rapid destruction of the epithelial barrier, and consequent fluid leakage and diarrhea. Once phagocytosed by the PMNs, *Shigella* cannot escape. This process keeps the bacteria localized, thus preventing submucosal invasion and subsequent systemic dissemination.

Shigella spp. also produce different types of toxins. The ShET1 is encoded by the 46 kb chromosomally located PAI called SHI-1. This toxin is an A1B5 toxin similar to the CT toxin of *V. cholerae* (see preceding). The ShET1 toxin is mainly found in *S. flexneri* 2a strains. The *Shigella* enterotoxin 2 (ShET2) is encoded by the pWR100 virulence plasmid and produced by most *Shigella* strains. Shiga toxin (STx) is a lethal cytotoxin encoded by the lambdoid bacteriophage that is integrated in the chromosome of *S. dysenteriae.* This toxin has an A1B5 structure and functions by cleaving the 28S rRNA of host cells and inhibiting protein synthesis, which leads to cell death. This toxin may also be responsible for the more severe infections that are typical of *S. dysenteriae* when compared to the other species.

Salmonella (*nontyphoidal serovars*)

Salmonella spp. are Gram-negative facultatively anaerobic rods. The genus comprises two species. One of the species, *S. enterica*, is further subdivided into six subspecies with approximately 2500 serovars (serotypes). *S. enterica* causes a variety of infections in diverse animal hosts including humans, domestic animals, and birds, with infections in humans being caused mostly by serovars in subspecies I. *S. enterica* serovars *typhimurium*, *enteritidis*, *newport* and *heidelberg,* referred to as nontyphoidal *Salmonella*, cause gastroenteritis (salmonellosis), whereas the serovars *typhi*, *paratyphi*, and *sendai* cause enteric fever (see the section following on *S. typhi*).

Nontyphoidal *Salmonella* infections occur at a high rate in both developed and developing countries. The nontyphoidal S*almonella* is the most common bacterial pathogen associated with food-borne outbreaks. The infection usually occurs after ingestion of contaminated water or foods of animal origin such as meat, poultry, eggs, and dairy products. The infectious dose in adult volunteers has been shown to be 10^6. Disease symptoms usually appear within 6–48 h of ingestion of the bacteria and include nausea, vomiting, abdominal cramps, and diarrhea. The disease is self-limiting and usually lasts no longer than 3–7 days, but can lead to serious complications in immunocompromised hosts such as HIV patients.

Salmonella infections develop in both the small and large intestines. Invasion is initiated by attachment of the pathogen to the intestinal epithelial cells by different types of fimbriae including type 1 fimbriae, long polar fimbriae (Lpf), and the thin aggregative fimbriae (curli). Subsequently, the bacteria either invade the intestinal epithelial cells or get internalized by the M cells of the Peyer's patches. Invasion of epithelial cells is mediated by TTSS and the secreted effector proteins encoded by the 40 kb *Salmonella* Pathogenicity Island 1 (SPI-1) located on the *Salmonella* chromosome. Like *Shigella* (see preceding), *Salmonella* enters host cells by a 'triggering mechanism' that involves cytoskeletal alterations resulting in the formation of actin-rich membrane ruffles that engulf the pathogen. The TTSS pore complex, SipB/SipD, and the SPI-1 effector proteins (SipA, SopB, SopD, and SopE/E2) then stimulate a host signaling cascade and cytoskeletal changes involving activation of host proteins such as Cdc42 and Rac. These events alter actin dynamics at the point of bacterial entry and subsequent bacterial uptake. *Salmonella* also produces flagellin, the monomeric subunit of the bacterial flagellae, and is able to translocate it into host cell cytosol using the SPI-1 TTSS. There, flagellin induces production of proinflammatory cytokines such as IL-8. Once intracellular, *Salmonella* remains trapped in a vacuolar compartment and, unlike *Shigella*, does not escape into the cytoplasm.

To cross the epithelial barrier, *Salmonella* can also use dendritic cells, the cells that are able to move between adjacent intestinal epithelial cells without disrupting the tight junctions. After translocation across the epithelial barrier, the nontyphoidal *Salmonella* induces a massive influx of neutrophils attracted by the secreted effector protein SipA. Bacteria become phagocytosed by macrophages where they use the SPI-1 SipB protein to induce a rapid proinflammatory cell death that has features of both apoptosis and necrosis. This also induces activation of proinflammatory cytokines IL-1 and IL-18, further participating in the inflammatory process within the intestine leading to increased fluid loss and diarrhea. Another TTSS of *Salmonella*, the SPI-2 TTSS, is also involved in the early and complete induction of intestinal inflammation (see '*S. typhi*' about SPI-2).

Campylobacter jejuni

Campylobacter species are Gram-negative, motile, microaerophilic, spirally curved rods. There are 14 species within the genus *Campylobacter*. Of these species, *C. jejuni* is the prototype for causing the enteric infection referred to as campylobacteriosis. *C. jejuni* has its habitat within the gastrointestinal tract of a broad spectrum of animals including domesticated animals and birds. Campylobacteriosis results from the consumption of contaminated water or foods, especially undercooked poultry, and raw milk. It may also spread in a fecal–oral fashion either from person to person or from animal to person. The organism preferentially grows at 42 °C and is sensitive to low pH, drying, freezing, and high temperature. The infectious dose varies depending on the source of bacteria. As few as 500 organisms were able to produce disease in one volunteer study. The incubation period varies from 1 to 7 days, while the symptoms of infection usually emerge within 3–4 days. Symptoms usually include fever, abdominal pain, and diarrhea, which is sometimes bloody. When symptoms of bloody diarrhea occur, the infection may be confused with idiopathic inflammatory bowel disease, either ulcerative colitis or Crohn's disease. The illness usually resolves in 7 days, but relapse can occur in untreated patients. Also, in rare cases, Guillain–Barré syndrome, an acute neuroparalytic disease, has been reported, usually within 2–3 weeks after the diarrheal illness. Postinfectious arthritis may also occur.

C. jejuni is among one of the most frequent bacterial causes of enteric infections in both industrialized and developing nations. It also causes travelers' diarrhea, ranking as the second cause after ETEC. In developing countries *C. jejuni* infections occur mainly in childhood before the age of five and primarily by person-to-person transmission. In these children the disease symptom is mostly self-limited watery diarrhea, which often leads to long-term asymptomatic carriage of the bacteria. However, in industrialized nations such as the United States and the United Kingdom, most of the disease outbreaks are food-borne and manifest as an inflammatory dysentery-like diarrhea in adults.

C. jejuni causes inflammation in both the small and large intestines. Other than primates, no animal model reproduces infections similar to humans. Most of the knowledge of the pathogenicity of this organism is derived from experimental infections in ferrets and chicks as well as from *in vitro* studies in intestinal epithelial cell lines such as INT 407, HEp-2, and Caco-2. In addition, sequencing of the relatively small genome of *C. jejuni* (i.e., 1.6 Mb) has helped in elucidating some of the potential virulence determinants.

A significant body of evidence from these *in vitro* and *in vivo* studies shows that *C. jejuni* colonizes the intestine by invading the intestinal epithelial cells using a unique mechanism. Once bacteria are ingested, they use their flagella and their corkscrew morphology to penetrate the intestinal mucosal barrier and reach the epithelial cells. There, they can bind to the cells via CadF, an adhesion protein that has been shown, *in vitro*, to bind to fibronectin on the basolateral side of the epithelial cells. The *C. jejuni* capsule, as well as the secreted protein, CiaB, and the flagellar protein, FlaC, have also been shown to be required for invasion of cultured epithelial cells. Unlike other pathogens such as *Shigella*, *Salmonella*, and *Yersinia* that use an actin microfilament-dependent mechanism for entry, *C. jejuni* uses microtubule polymerization during the invasion process. Upon bacterial contact with the epithelial cells, the cell membrane forms specialized structures known as pseudopods, which contain microtubules and are able to capture the bacteria. Following internalization, the bacterial-containing vacuoles move along the microtubules toward the cell nucleus. In addition, it is thought that the CdtA and CdtC components of the cytolethal distending toxin (CDT) might mediate binding of bacteria to the host cell receptors and cause subsequent internalization of bacteria through clathrin-mediated endocytosis, the natural process used by mammalian cells for nutrient uptake.

The mechanism of intracellular survival and growth of *C. jejuni* is not yet understood. CdtB, the toxic moiety of CDT, is thought to act as a DNase and cause DNA damage in the nucleus and, thus, cytotoxicity. Moreover, CDT induces secretion of the proinflammatory cytokine IL-8 from the epithelial cells, leading to recruitment of inflammatory cells. CDT also induces apoptosis in monocytic cell lines. Another inducer of proinflammatory response is JIpA, an adhesin protein essential for the binding of bacteria to cultured HEp-2 cells.

C. jejuni also carries hypervariable sequences within its genes that encode for the proteins required for capsule and lipooligosaccharide (LOS) biosynthesis. These hypervariable sequences probably play a role in immune avoidance and increased survival of the bacterium.

Locoregional Invasion

Yersinia *spp.*

Yersinia spp. are Gram-negative, aerobic rods. The three species that are pathogenic for humans include *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* is the etiologic agent of the bubonic plague (the 'Black Death'), which is thought to have killed one-fourth of the European population during medieval times. *Y. pseudotuberculosis* and *Y. enterocolitica* are the enteropathogenic species, the latter being the main cause of yersiniosis. Both species have reservoirs in a wide range of animals including rodents, birds, farm animals, and pets. Transmission of infection takes place by the fecal–oral route and usually by ingestion of contaminated water or foods such as milk and raw pork. The organisms grow at a wide range of temperature from 4 °C to 37 °C, thus enabling them to multiply even after refrigeration.

Yersiniosis is a disease that is mostly caused by *Y. enterocolitica* and is relatively common in developed parts of the world such as Europe, especially among children and young adults. The infecting dose for the organism is high (10^9 organisms) and the symptoms of the disease appear after 4–7 days of incubation. The symptoms, which can last for 1 day to 4 weeks, are enteric fever-like and include fever, abdominal pain, and diarrhea. Mesenteric adenitis can also occur, and this can be confused clinically with acute appendicitis. Extraintestinal syndromes can result from yersiniosis that may include septicemia and postinfectious arthritis. On the other hand, *Y. pseudotuberculosis* mainly causes mesenteric adenitis and diarrheal syndrome is less frequent.

Following ingestion, *Yersinia* cross the intestinal epithelium through the M cells within the Peyer's patches of the ileum. *Yersinia* entry takes place by the so-called zippering' mechanism that involves an intimate contact between the bacterium and the host cell. This is achieved by the binding of invasin (Inv), a chromosomally encoded 103 kDa outer membrane protein of *Yersinia*, to the $\beta 1$ integrins that are also expressed on the apical surface of the M cells. The dynamics of binding cause integrin clustering and transmission of intracellular signals, which leads to the formation of focal adhesion complexes, rearrangement of the actin cytoskeleton, and bacterial internalization. Other *Yersinia* surface proteins such as *Yersinia* adhesin A (YadA) and attachment–invasion locus (ail) also contribute to the invasion process.

Invasion leads to the production of proinflammatory cytokines and massive infiltration of neutrophils. However, *Yersinia* resists being phagocytosed by neutrophils and resident macrophages. The antiphagocytosis function can be mediated by the injection of the plasmid-encoded effector proteins YopH, YopT, YopE, and YpkO/YopO through the plasmid-encoded TTSS called Ysc. Secretion of the Yop proteins is regulated by temperature, calcium concentration, and contact with the host cell. Following injection, YopH, which is a homologue of mammalian tyrosine phosphatase, dephosphorylates macrophage proteins such as FAK and paxillin. YopT, YopE, and YpkO/YopO modify macrophage proteins including Rac1, Rho, and Cdc42. Moreover, YopJ/YopP induces apoptosis of macrophages. This way, Yops inhibit the phagocytic activity of macrophages and therefore the bacteria remain extracelluar. Bacteria also invade epithelial cells by the Inv-mediated entry and trigger the expression of a variety of cytokines such as interleukin IL-8. Subsequently, polymorphonuclear leukocytes (PMNs) are recruited to the site of infection, leading to the formation of microabscesses in the intestine. Bacteria also disseminate through the lymphatic system and form microabscesses in mesenteric lymph nodes, leading to clinical manifestations of mesenteric adenitis.

Y. entrocolitica also produces an enterotoxin similar to the heat-stable toxin of *E. coli*, called Yst. It is postulated that this toxin may play a role in the food poisoning caused by this pathogen.

Locoregional Invasion Followed by Systemic Dissemination

Salmonella typhi

Salmonella enterica serovar typhi (*S. typhi*) is the etiologic agent of typhoid fever, a severe systemic illness affecting humans. The disease is transmitted by ingestion of contaminated drinking water or by close contact with the infected individuals or with individuals that are merely carriers. The disease is characterized by fever and abdominal symptoms, which emerge after a long incubation period of 1–2 weeks. Diarrheal symptoms mainly occur in immunocompromised individuals. The disease can resolve or turn into a chronic carrier stage with relapse. Typhoid fever is prevalent in Southeast Asia where patients often suffer because of recurrent infections.

Studies on typhoid fever have been restricted due to the fact that the human species is the only known reservoir for *S. typhi*. However, *S. typhimurium*, which normally causes salmonellosis in humans, can colonize the small and large bowel of mouse and cause a typhoid-like systemic illness in this animal. For these reasons, most of the understanding of mechanisms of pathogenesis of typhoid fever is based on infection of susceptible mice with *S. typhimurium*.

S. typhi has gained the pathogenic ability to penetrate the intestinal submucosa, and further multiply in lymphatic and reticuloendothelial cells. Upon ingestion, the pathogens colonize the ileum and cecum, followed by translocation through the intestinal mucosa by invading the specialized epithelial M cells of Peyer's patches and by also employing the dendritic cells. These events involve a complex process including the actions of both SPI-1 and SPI-2 TTSSs and their effector proteins. This process is similar to the invasion mechanism used by nontyphoidal *Salmonella* (see preceding). The bacteria are then carried from the intestine into the mesenteric lymph nodes and further within the blood circulation via CD18-expressing mononuclear phagocytes, in which bacteria can survive and multiply. Through the blood, bacteria are carried to systemic sites where they are taken up by interstitial macrophages present in bone marrow, liver, and spleen tissues. The ability of the bacteria to survive and replicate within macrophages is central for the pathogenesis and systemic dissemination of the infection. This ability is conferred by the various effectors of SPI-1 and SPI-2 such as SipA, SpiC, and SseD. These effectors assist the pathogen in remaining within a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV), which is distinct from phagosomes or lysosomes. Survival within the macrophages is also mediated by the PhoP/PhoQ two-component regulatory system.

Another pathogenic determinant of *S. typhi* that induces inflammatory responses in macrophages is LPS. *S. typhi* strains also produce a polysaccharide capsule called the Vi capsule, which is encoded by SPI-7. It has been postulated that the Vi capsule modulates the infiltration of PMNs within the intestinal lumen at the early stages of colonization.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, nonsporulating, facultatively anaerobic rod. It is the only *Listeria* species that causes disease in humans. The organism is ubiquitous in nature and is found in soil, water, and as a commensal in many animals. Infections with *L. monocytogenes*, termed listeriosis, are usually food-borne. The infection is caused by ingestion of a dose of more than 10^9 organisms in contaminated foods such as soft cheese, raw milk, raw vegetables, and processed meat. The organism can grow in acidic and high-salt environments and survive for long periods if refrigerated. Listeriosis has a long incubation period that ranges from 11 to 70 days. The symptoms of gastrointestinal disease include nausea, vomiting, and diarrhea, which are usually self-limiting in normal healthy people. However, in specific high-risk groups such as neonates, the elderly, pregnant women, and other immunocompromised hosts, more serious manifestations such as bacteremia, meningitis, abortion, and stillbirth can occur.

L. monocytogenes colonizes the small intestine by invasion of the enterocytes. The organism can also disseminate into the blood circulation and infect other organs such as the liver and brain. As with *Yersinia*, entry of *L. monocytogenes* into the epithelial cells takes place by the 'zippering' mechanism, which is an actin-mediated phagocytosis process (see preceding). Two surface proteins, internalin A (InlA) and internalin B (InlB), bind to the host cell receptors Met and *E*-cadherin, respectively, and this binding leads to activation of a complex set of signaling pathways resulting in cytoskeletal rearrangements with consequent bacterial internalization. Internalization of *L. monocytogenes* can also be mediated by a clathrin-dependent endocytosis pathway.

Following internalization, bacteria lyse the phagosomal vacuole and escape into the cytosol by secreting a pore-forming toxin, the listeriolysin O (LLO), and two phosphatases, PlcA and PlcB. Within the host cytoplasm, bacterial protein Act-A induces polymerization of actin to propel itself through the cytoplasm and spread to the adjacent cells in a manner similar to *Shigella* (see preceding). The mechanism of intracellular growth at that point is not well understood. However, recent transcriptomic studies show that approximately 500 genes are involved in this mechanism. One protein shown to play a role in growth inside the host cytosol is Hpt, a sugar update protein. In addition, several two-component regulatory proteins have also been identified by genomic sequencing. These proteins possibly contribute to intracellular bacterial survival.

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Escherichia Coli

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Defining Statement
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Glossary

enterobacteriaceae A family of the γ-(*Greek gamma*) Proteobacteria that includes *Escherichia coli* and related Gram-negative bacteria.

enterohemorrhagic E. coli (EHEC) A bacterium that causes hemorrhagic colitis.

enteropathogenic E. coli (EPEC) A bacterium that causes diarrhea after colonization of the mid-distal small intestine.

enterotoxigenic E. coli (ETEC) The bacterium that is the most common agent of watery ‘tourist’s’ diarrhea.

hemorrhagic colitis Bloody diarrhea caused by enterohemorrhagic *E. coli* (EHEC).

Abbreviations

ATP	adenosine triphosphate
EAggEC	enteroaggregative *E. coli*
ECA	enterobacterial common antigen
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
HGT	horizontal gene transfer
LEE	locus of enterocyte effacement
LPS	lipopolysaccharide
LT	heat labile
SLT	Shiga-like toxins
ST	heat stable

Defining Statement

Escherichia coli is the best studied cellular form of life and continues to serve as one of the principal model systems in Biology. Much has been elucidated regarding its structure, metabolism, and genetics. The species includes a large number of strains that differ considerably in genomic composition and pathogenic potential. Many of these are innocuous commensals of mammals, others cause serious disease in both animals and humans.

Introduction

Escherichia coli is a Gram-negative facultative anaerobic non-spore-forming motile rod. The species belongs to the Enterobacteriaceae family of the γ-(*Greek gamma*) Proteobacteria and includes a large number of strains that differ in pathogenic potential. Certain strains are common innocuous residents of the intestine of mammals; others cause human and animal infections of the digestive and urinary tracts, blood, and central nervous system. The structure, biochemical functions, and genetics of this organism are well studied, making it the best known of all cellular forms of life. This organism has occupied center stage in the development of molecular biology.

Taxonomy

E. coli is one of eight or more recognized species of the genus *Escherichia*. The genus is named after Theodor Escherich, who first isolated *E. coli* in 1884. Historically, the species is defined on the basis of certain readily measurable biochemical activities shared by most strains. Thus, *E. coli* generally ferments lactose, possesses lysine decarboxylase, produces indole, does not grow on citrate, does not produce H_2S, and is Voges–Proskauer negative (does not produce acetoin). The chromosomal DNA of strain K-12 is 50.8% GC. The limits of the taxonomic definition are under scrutiny because DNA hybridization data suggest that, contrary to tradition, *Escherichia* and *Shigella* belong to the same genus. *E. coli* comprises a number of strains that share the same basic taxonomic features, with about 70% DNA homology at the extremes. At the time of writing, the genomes of seven strains have been completely sequenced.

E. coli strains are defined mainly by their antigenic composition. Of taxonomic relevance are over 170 different serological types of lipopolysaccharide antigens (O antigens) and 80 types of capsular (K antigens). Other properties that are used to define individual strains are H antigens (flagellar proteins), F antigens (fimbrial proteins), and phage and colicin sensitivity.

Quantitative approaches to defining taxonomic relationships have been based on patterns of isozymes of metabolically important enzymes, protein composition of the outer membrane, and increasingly genomic sequences. Although there is a huge number of combinations of these properties, the variety of strains that have been isolated is circumscribed (perhaps in the thousands). By the criteria used, most of the strains in today's world appear to be the clonal descendants of relatively few ancestors. Genetic exchange leading to recombinational events thus seems to be an infrequent event in the environment. It has been estimated that, for this species, major episodes of selection occur once in 30 000 years.

Ecology

E. coli is the most abundant facultative anaerobe in the feces, and therefore the colon, of normal humans and many mammals. It is commonly present in concentrations of $\sim 10^7$–10^8 live organisms per gram of feces. Thus, the total number of individual *E. coli* cells present on Earth at any one time exceeds 10^{20} and their total weight 10^2 metric tons.

E. coli is far from the most abundant organism in the colon and is outnumbered 100-fold or more by strict anaerobes. *E. coli* and other intestinal facultative anaerobes colonize not only in the large intestine of vertebrates but also in the ileum, the distal segment of the small intestine. In the ileum, *E. coli* is present in numbers that approximate those of the organisms in feces. The ileal population is transient, being rapidly propelled into the cecum by peristalsis. These organisms are apparently derived via a reflux mechanism from the cecum and are rarely acquired anew by ingestion. A better understanding of what shaped *E. coli* during evolution will require a systematic study of the distinct selective pressures it may have faced in these two very different regions of the intestine, the ileum and the colon. In the ileum, *E. coli* may have been selected for rapid growth under oxygenated conditions; in the colon, in competition for limited nutrients and in the presence of noxious chemicals under anoxic conditions.

Most fecal *E. coli* isolates are well adapted to colonizing the mammalian intestine and seldom cause disease. When human strains are cultivated in the laboratory, they tend to lose the ability to colonize. Included among these is K12, the most widely used strain in the molecular microbiology.

E. coli cells are periodically deposited from their intestinal residence into soils and waters. It has been thought that they do not survive for extended periods of time in such environments and could be cultured only for a few days (seldom weeks) after their introduction. For this reason, their presence has been taken as a measure of recent fecal contamination, and the coliform count of the drinking water supply or of swimming facilities is still a common measure of microbiological water purity. The notion that *E. coli* has a short survival time in the environment has been challenged, and newer work suggests that the presence of these organisms may not be a reliable indication of recent fecal pollution. Mammals become colonized with *E. coli* within a few days of birth, possibly from the mother or other attendants. How the organisms are transmitted to the neonate is not known with certainty; this may occur during passage through the birth canal or, shortly after birth, via the fecal–oral route.

Structure and Function of Cell Parts

In both structure and function, *E. coli* serves as the prototype for members of the Enterobacteriaceae. An example of the overall composition of this organism in its growth phase is shown in **Table 1**.

Fimbriae (Pili)

E. coli strains carry one or two kinds of fimbriae, common, and conjugative (sex pili). Common fimbriae are usually found in numbers of 100–1000 per cell and consist mainly of an acidic hydrophobic protein called fimbrin. The common fimbriae fall into seven groups according to the amino acid sequence of their major fimbrin. Fimbriae are highly antigenic, comprising many F antigens.

E. coli strain K12 possesses only type 1 common fimbriae. This strain and others alternate between the fimbriated and nonfimbriated condition, a phenomenon known as phase variation. It is thought that the presence of common fimbriae allows organisms in their first efforts to colonize their host by attaching to epithelial cells. Inside the body, turning off the synthesis of common fimbriae may lessen the chances that the organisms will be phagocytized by white blood cells.

The sex fimbriae (usually called pili) are encoded by plasmids such as F or R and are usually present in one or a

Table 1 Overall macromolecular composition of an average *Escherichia coli* cell

Number of macromolecule	*Percentage of total dry weight*	*Weight per cell ($10^{-15} \times g$)*	*Molecular weight*	*Molecules per cell*	*Different kinds of molecule*
Protein	55.0	155.0	4.0×10^4	2 360 000	1050
RNA	20.5	59.0			
23S ribosomal RNA		31.0	1.0×10^6	18 700	
16S ribosomal RNA		16.0	5.0×10^5	18 700	
5S ribosomal RNA		1.0	3.9×10^4	18 700	
Transfer RNA		8.6	2.5×10^4	205 000	60
Messenger RNA		2.4	1.0×10^9	1380	400
DNA	3.1	9.0	2.5×10^9	2.13	
Lipid	9.1	26.0	705	22 000 000	
Lipopolysaccharide	3.4	10.0	4346	1 200 000	
Murein	2.5	7.0			
Glycogen	2.5	7.0	1.0×10^6	4360	
Total macromolecules	96	273.0			
Soluble pool	2.9	8.0			
Building blocks		7.0			
Metabolites, vitamins		1.0			
Inorganic ions	1.0	3.0			
Total dry weight	100.0	284			
Total dry weight per cell		2.8×10^{-13} g			
Water (at 70% of cell)		6.7×10^{-13} g			

These values are for *E. coli* in balanced growth in a glucose-minimal medium at 37 °C. Reproduced from Neidhardt FC, Ingraham JL, and Schaechter M (1990) *Physiology of the Bacterial Cell*. Sunderland, MA: Sinauer Associates.

few copies per cell. These structures cause the donor and recipient bacteria to make contact, allowing the transfer of DNA during conjugation.

Flagella

E. coli is usually endowed with only 5–10 flagella per cell. However small, this complement suffices to endow the cells with brisk motility. The flagella are typically 5–10 µm (*Greek mu*)-long and are arranged randomly around the cell surface, a pattern called peritrichous flagellation. As is typical of bacterial flagella, those of *E. coli* are composed of a long filament, a hook, and a basal body. The principal component of *E. coli* flagella is an *N*-methyl-lysine-rich protein known as flagellin. Its size, usually around 55 kDa, varies among strains. Around 20 000 subunits of this protein assemble to make the flagellar filament. *In vitro*, flagellin self-assembles into flagella-like filamentous cylindrical lattices with hexagonal packing.

The *E. coli* flagellar genetic system consists of about 40 genes arranged in five regions. These genes are involved in structure, function, assembly, and regulation. Flagella are highly antigenic, comprising a large number of H antigens. The N- and C-termini of various H antigens are highly conserved, the major antigenic divergence being found in the central region of the molecule.

Capsule and Outer Membrane

In some strains, the outer membrane of *E. coli* covered by a polysaccharide capsule composed of K antigens. Other polysaccharides, the M antigens (colanic acids, which are polymers of glucose, galactose, fucose, and galacturonic acid), are synthesized under conditions of high osmolarity, low temperature, and low humidity, suggesting that normally these compounds may be made in response to stressful conditions in the external environment. In addition, *E. coli* and other enteric bacteria possess a glycolipid anchored in the outer leaflet of their outer membrane, called the enterobacterial common antigen (ECA).

The outer membrane of *E. coli* is typical of that of Gram-negative bacteria, consisting of a lipid bilayer whose inner leaflet is made up largely of phospholipids and whose outer leaflet is made of lipopolysaccharide (LPS). Interspersed are several kinds of membrane proteins. One, the murein lipoprotein, is small (7.2 kDa) and exists in 7×10^5 copies per cell. This protein contains three fatty acid residues that help anchor it into the inner leaflet of the outer membrane, whereas the rest of the molecule is located in the periplasm. About one-third of the molecules are covalently linked to the cell-wall peptidoglycan. The major outer-membrane proteins include the pore-forming proteins, called porins Omp C, Omp F, and Pho E. Together, these porins are present in about 10^5 copies per cell. Their sizes vary from 36.7–38.3 kDa. The diameters of the pores are 1.16 nm for Omp F and Pho E and 1.08 nm for Omp C. The synthesis of these porins is regulated by environmental conditions; thus, Omp F is repressed by high osmotic conditions or high temperature, Omp C is derepressed by high osmotic conditions, and Pho E is synthesized when cells are starved for phosphate. These findings suggest that the organisms use narrower porin channels in the animal host than in the outside environment, which invites speculation about the nature of chemical and metabolic challenges faced under the two conditions.

Certain compounds that are too large to diffuse through *E. coli* porin channels are carried across the outer membrane by special transport proteins. These compounds include maltose oligosaccharides, nucleosides, various iron chelates, and vitamin B_{12}. The proteins involved, as well as the porins, also act as receptors for the attachment of bacteriophage and colicins.

Periplasm and Cell Wall

By functional tests of solute partition and electron microscopy, the periplasm (the space between the inner and outer membranes) of *E. coli* makes up 20–40% of the cell volume. This compartment is osmotically active, in part because it contains large amounts of membrane-derived oligosaccharides (molecules of 8–10 linked glucose residues substituted with 1-phosphoglycerol and O-succinyl esters). There is evidence that the contents of the periplasmic space form a gel. The *E. coli* periplasm contains over 60 known proteins, including binding proteins for amino acids, sugars, vitamins, and ions; degradative enzymes (phosphatases, proteases, and endonucleases); and antibiotic detoxifying enzymes (β-lactamases, alkyl sulfodehydrases, and aminoglycoside phosphorylating enzymes). The periplasmic environment is oxidizing, whereas that of the cytoplasm is reducing. This explains why certain secretory proteins that require disulfide bonds for activity are inactive in the cytoplasm.

As in most bacteria, the cell wall of *E. coli* consists of a peptidoglycan layer responsible for cell shape and rigidity. In this organism, peptidoglycan is one or, at most, a few molecules thick. It is anchored to the outer membrane at some 400 000 sites via covalent links to the major membrane lipoprotein and noncovalent links to porins.

Evidence indicates that the periplasm is spanned by 200–400 adhesion zones between the outer membrane and the cytoplasmic membrane. These appear to be the sites of attachment of certain bacteriophage and of export of outer-membrane proteins and lipopolysaccharide. In addition to these apparently scattered junctions, the two membranes appear joined at defined periseptal annuli, ring-shaped adhesion zones that are formed near the septum.

Cytoplasmic Membrane

The cytoplasmic membrane of *E. coli* is made up of about 200 distinct proteins and four kinds of phospholipids. Proteins make up about 70% of the weight of the structure. Under aerobic conditions, the *E. coli* cytoplasmic membrane contains a number of dehydrogenases (e.g., NADH-, D- and L-lactate, and succinate dehydrogenases), pyruvate oxidase, cytochromes (of the o and d complexes), and quinones (mainly 8-ubiquinone). Anaerobically grown *E. coli* may contain other dehydrogenases (e.g., formate and glycerol-3-phosphate dehydrogenases) and enzymes involved in anaerobic respiration (nitrate and fumarate reductases).

The cytoplasmic membrane is the site of adenosine triphosphate (ATP) synthesis. The cytoplasmic membrane systems involved in the transport of solutes are highly efficient and permit this species to grow in relatively dilute nutrient solutions. The cytoplasmic membrane of *E. coli* contains over 20 proteins involved in various aspects of peptidoglycan biosynthesis, cell wall elongation, and cell division. About 10 of these proteins have been identified by their ability to covalently bind β-lactam antibiotics. They are known as the penicillin-binding proteins, and some have been shown to be involved directly in cell-wall synthesis.

Cytoplasm

Most of the thousands of biochemical reactions necessary for the growth of *E. coli* take place in the cytoplasm. These activities are divided into those concerned with metabolic fueling (production of energy, reducing power, and precursor metabolites), biosynthesis of building blocks, polymerization into macromolecules, and assembly of cell structures. For *E. coli*, each of these activities is generally the same as for all microorganisms, but with certain species specific characteristics.

In fast-growing *E. coli*, much of the cytoplasmic space is taken up by ribosomes. The number of ribosomes per cell is proportional to the growth rate, ranging from about 2000 in cells growing at 37 °C at doubling rates of 0.2 h^{-1} to >70 000 at a doubling rate of 2.5 h^{-1}, where they make up about 40% of the cell mass. In *E. coli*, the genes for the four ribosomal RNAs (16S, 23S, 5S, transfer) are arranged in seven operons located at different sites on the chromosome. Most of these operons are found near the origin of chromosome replication and, thus, are replicated early. This arrangement ensures that ribosomal RNAs are made in large amounts during rapid growth. Each operon encodes one, two, or three transfer RNAs at sites between the 16S and 23S RNA genes or at the end of the operon. The 52 ribosomal proteins are encoded by 21 transcriptional units. Ribosomal RNAs and proteins assemble into particles via a precise sequence of reactions that has been well studied *in vitro*.

DNA and Nucleoid

The entire sequence of several strains of the *E. coli* genome has been determined. That of the K12 strain consists of 4 639 679 bp and codes for an estimated 4460 proteins (**Table 2**) in about 3277 transcriptional units and 175 RNA genes. About three quarters of the genes have been assigned a function, but this is a changing figure. Most of these genes have been annotated based on experimental data, the highest proportion for any organism. About 20% of the genes appear to have been acquired by horizontal gene transfer (HGT) from other organisms. Protein-coding sequences account for about 88% of the genome, stable RNAs for 0.8%, and noncoding repeats for 0.7%, leaving about 10% of the genome for regulatory and other functions. Most transcriptional units contain a single gene and very few have more than six. The genome contains a number of repeated sequences, most of unknown function, as well as several dozen insertion sequences, cryptic prophage, and phage remnants. Some pathogenic strains of *E. coli* contain about 1 million more base pair than the K-12 strain. These extra genes were probably acquired by HGT.

As in many bacteria, the DNA of *E. coli* consists of a circular molecule with a replicative origin and a terminus about 180° away. By genetic manipulations, the DNA can be linearized, apparently without undue effects on the physiology of the cells.

The nucleoid of *E. coli* is a highly lobular intracytoplasmic region, generally located toward the center of the cell. In this region, the DNA is found at a local concentration of 2–5% (w/v). The reason why this long molecule is folded and physically limited to the nucleoid region is not well understood. It is known that *in vivo* the DNA is negatively supercoiled into some 50 individual domains. Nucleoids of superhelicity and dimension similar to those seen intracellularly can be isolated by the gentle breakage of cells in the presence of divalent cations.

Transcription takes place at the nucleoid–cytoplasm interface, as the nucleoid is thought to form a significant barrier to the diffusion of many macromolecules. The reason for the highly irregular shape of the nucleoid may be to contribute to the availability of genes for transcription. At least four small-molecular-weight proteins that bind to DNA are known to play a role in transcription, recombination, and replication. These nucleoid-associated proteins range in molecular weight from 9200 to 15 400. Two, HU and IHF, are among the abundant *E. coli* proteins and are present in 20–50 000 monomers per cell.

Table 2 Genes of *Escherichia coli* (strain K12)

Total number of genes	4580
Genes for transport functions	484
Genes for metabolism	1596
Genes determining cell structure	840

The initiation of DNA replication takes place at a specific origin site, *oriC*, and is under the influence of a protein that is highly conserved among many bacteria, DnaA. Once initiated, DNA replication takes place at a nearly constant rate in moderately fast and fast-growing *E. coli*, until it reaches a terminus. The doubling of the chromosome takes 40 mm at 37 °C, which requires that the time of initiation of chromosome replication takes place before the end of the previous round in cultures growing faster than this.

Little is known about the mode of segregation of the nucleoids. The process takes place with considerable fidelity and, thus, cannot result from partitioning into progeny cells by chance alone. A widespread view is that the chromosome is attached to the cell membrane and that movement of the membrane serves as a primitive mitotic apparatus. However, this view is based largely on cell fractionation studies and is not supported by functional tests. At least *in vitro*, recently replicated (hemimethylated) origin DNA binds to the membrane with great specificity.

Metabolism and Growth

Biosynthetic and Fueling Reactions

The central metabolism of *E. coli* is carried out via the Embden–Meyerhof–Parnas pathway, the pentose pathway, and the tricarboxylic acid cycle plus, for the metabolism of gluconate, the Entner–Doudoroff pathway. As a facultative anaerobe, *E. coli* meets its energy needs by either respiratory or fermentative pathways. *E. coli* carries out a mixed acid fermentation of glucose that results in the formation of a large number of products. Under anaerobic conditions, the main products (and the moles formed per 100 moles of glucose used) are formate (2.4), acetate (37), lactate (80), succinate (12), ethanol (50), 2,3-butanediol (0.3), CO_2 (88), and H_2 (75).

The need for biosynthetic building blocks is met in *E. coli* by the production of 12 precursor metabolites common to all bacteria. The energy requirements for the manufacture of the major building blocks are shown in **Table 3**.

The precursor metabolites do not contain nitrogen or sulfur, which must enter the metabolic circuit independently. *E. coli* does not fix dinitrogen gas, but can use a number of compounds as a source of nitrogen, including ammonium ions and various amino acids. It can use nitrate and nitrite as terminal electron acceptors during anaerobic respiration by activating nitrate or nitrite reductases. However, under anaerobic conditions, no energy is generated by this process, and a source of reduced nitrogen is necessary for the anaerobic growth of *E. coli*. The incorporation of ammonium ion into

Table 3 Energy requirements for polymerization of the macromolecules in 1 g of cells

Macromolecule	*Amount of energy required* (μ*mol P*)
From activated building blocks	
DNA from dNTPs	136
RNA from NTPs	236
Protein from aminoacyltransfer RNAs	11 808
Murein, in part from activated building blocks	138
Phospholipids, in part from activated building blocks	258
Lipopolysaccharide	0
Polysaccharide (glycogen)	0
Total energy	12 576
From unactivated building blocks	
DNA from dNMPs	336
RNA from NMPs	1516
Protein from amino acids	21 970
Murein, in part from activated building blocks	138
Phospholipids, in part from activated building blocks	258
Lipopolysaccharide	0
Polysaccharide (glycogen)	0
Total energy	24 218

Reproduced from Neidhardt FC, Ingraham JL, and Schaechter M (1990) *Physiology of the Bacterial Cell*. Sunderland, MA: Sinauer Associates.

organic compounds is catalyzed either by L-glutamate dehydrogenase when ammonia is abundant or by glutamine synthetase and glutamate synthase, acting together, when ammonia is limiting.

The common sources of sulfur for *E. coli* are sulfate and sulfur-containing amino acids. Sulfate is transported into the cell after being reduced to H_2S by a sulfite reductase. Sulfur is then assimilated from H_2S using *O*-acetylserine sulfohydrolase to produce L-cysteine. *E. coli* has a complete system of transporting and using organic phosphates, including an inducible alkaline phosphatase in its periplasm.

Nutrition and Growth

E. coli is a chemoheterotroph capable of growing on any of a large number of sugars or amino acids provided individually or in mixtures. Some strains found in nature have single auxotrophic requirements, among them thiamin is common. The growth of many strains is inhibited by the presence of single amino acids, such as serine, valine, or cysteine. *E. coli* grows faster with glucose than with any other single carbon and energy source and reaches a doubling time of 50 min under well-oxygenated conditions at 37 °C. Doubling times with less favored substrates may be hours in length. Slow rates of growth can also be achieved by using an externally controlled continuous culture device or by adding a metabolic analogue and its antagonist to the culture at proper ratios. When the medium is supplemented with building blocks such as amino acids, nucleosides, sugars, and vitamin precursors.

E. coli grows more rapidly in rich-nutrient broths, reaching doubling times of 20 min at 37 °C. *E. coli* can grow at temperatures between 8 and 48 °C, depending on the strain and the nutrient medium. Its optimum growth temperature is 39 °C. *E. coli* does not grow in media containing a NaCl concentration greater than about 0.65 M. In response to changes in the osmotic pressure of the medium, *E. coli* increases its concentration of ions, especially K^+ and glutamate. The pH range for growth is between pH 6.0 and 8.0, although some growth is possible at values approximately 1 pH unit above and below this range.

Pathogenesis

Strains of *E. coli* are responsible for a large number of clinical diseases. In their manifestation, some of these diseases overlap with those caused by other species (e.g., *Shigella* and *Salmonella*). The most common infections caused by *E. coli* involve the intestinal and urinary tracts of humans and other mammals, where they produce simple watery diarrhea or locally invasive forms of infection (e.g., dysentery). *E. coli* infects deeper tissues, including the blood (septicemia) as a complication of focal extraintestinal infections such as pyelonephritis and, additionally, the meninges in newborns. The organism also causes mastitis in cattle. Strains of *E. coli* that produce intestinal infections are divided into groups according to the clinical picture they produce and their known virulence factors (**Table 4**). These strains are denoted by abbreviations (e.g., ETEC and EPEC, where the terminal EC stands for *E. coli*) and are proliferating. Infections by *E. coli* involve a large number of virulence factors, including toxins, adhesions, invasins, antiphagocytic surface components, and others.

Table 4 Classification of pathogenic *Escherichia coli*

Group	*Symptoms*
Enterotoxigenic *Escherichia coli* (ETEC)	Watery diarrhea ('travelers' disease')
Enteropathogenic *E. coli* (EPEC)	Watery diarrhea
Enterohemorrhagic *E. coli* (EHEC)	Bloody diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome, thrombocytopenic purpura
Enteroinvasive (EIEC)	Bloody diarrhea
Enteroaggregative (EAggEC)	Watery diarrhea, persistent diarrhea

Enterotoxigenic Strains

Enterotoxigenic *E. coli* (ETEC) strains acquired from food or water contaminated with human or animal feces are the most common bacterial agents of diarrhea in the United States and Europe. These strains circulate among a population, but the majority of people (especially adults) usually remain asymptomatic, most likely due to immunity afforded by previous exposure. ETEC strains are responsible for the 'tourist's diarrhea' that frequently affects persons traveling to countries with a low level of sanitation. Watery diarrhea due to *E. coli* resembles that seen in mild cases of cholera.

ETEC strains colonize the small intestine, where they produce one or both of two enterotoxins called heat labile (LT) and heat stable (ST). Both toxins act by changing the net fluid transport activity in the gut from absorption to secretion. LT is structurally similar to cholera toxin and activates the adenylate cyclase–cyclic adenosine monophosphate system, whereas ST works on guanylate cyclase. The intestinal mucosa is not visibly damaged, the watery stool does not contain white or red blood cells, and no inflammatory process occurs in the gut wall. Gut cells activated by LT or cholera toxin remain in that state until they die, whereas the effects of ST on guanylate cyclase are turned off when the toxin is washed away from the cell.

Enteropathogenic Strains

Enteropathogenic *E. coli* (EPEC) strains cause watery and sometimes bloody diarrhea after the colonization of the mid-distal small intestine (ileum). They recognize their preferred hosts and tissues by means of plasmid-encoded surface adhesins specific for receptors on the intestinal brush-border membranes, called the bundle-forming pili (or fimbriae). Characteristic of EPEC infection is an attachment–effacement (A/E) lesion on the surface of enteric epithelial cells. The affected cells form a broad flat pedestal (effacement) beneath the attached microorganism, which, by damaging the absorptive surface (villi), contributes to the diarrhea. The genes required for the formation of A/E lesions are located on a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE). Once bound to the epithelial cells, EPEC export critical virulence factors by a type III secretion apparatus, causing several host signals to be activated.

Enterohemorrhagic Strains

The enterohemorrhagic *E. coli* (EHEC) comprise a limited number of serotypes that cause a characteristic nonfebrile bloody diarrhea known as hemorrhagic colitis. The most common of these serotypes in the United States is O157:H7, whereas others, particularly O26, are found with greater frequency elsewhere in the world. *E. coli* O157:H7 causes both outbreaks and sporadic disease. The organism is commonly isolated from cattle and other ruminants, and several outbreaks have been traced to undercooked hamburger meat. EHEC strains have two special characteristics of pathogenic importance. First, they produce high levels of two related cytotoxins that resemble toxins of *Shigella*, with the same protein synthesis-inhibitory action and binding specificity. These toxins are therefore called Shiga-like toxins (SLT) I and II. The SLT are cytotoxic for endothelial cells in culture. Second, they possess a gene highly homologous to the EPEC attaching and effacing pathogenicity island. In combination, the proteins encoded by this gene and the SLT presumably damage the gut mucosa in a manner characteristic of hemorrhagic colitis.

EHEC strains cause systemic manifestations (hemolytic–uremic syndrome or thrombotic thrombocytopenic purpura in adults) that are believed to be related to systemic absorption of SLT, possibly in combination with endotoxin, which up-regulates the expression of the SLT receptor on host cells. These syndromes represent the clinical response to endothelial damage of glomeruli and the central nervous system. Organ damage is sometimes permanent.

Other Strains that Cause Intestinal Infections

Enteroinvasive *E. coli* (EIEC) strains cause dysentery, resembling that due to *Shigella*. Unlike the strains already described, which are noninvasive, EIEC strains are selectively taken up into epithelial cells of the colon, requiring for this process a specific outer-membrane protein. EIEC strains also make Shiga-like toxins. Cell damage by these strains triggers an intense inflammatory response. Other strains, called enteroaggregative *E. coli* (EAggEC), are associated with diarrhea in infants under 6 months of age, often persisting for weeks with marked nutritional consequences. EAggEC strains spontaneously agglutinate (aggregate) in tissue culture.

Strains that Infect the Genitourinary Tract

E. coli strains are the most common cause of genitourinary tract infections in humans, including cystitis, pyelonephritis, and prostatitis. Strains that cause pyelonephritis (UPEC) usually have specific O and K antigens, and are often hemolytic by virtue of encoding an RTX hemolysin. They also possess unique fimbriae (pili) that bind specifically to a membrane glycolipid of kidney tissue. These structures are called P pili because the receptor is a complex galactose-containing molecule that is part of the P blood group antigen. About 1% of humans are P antigen-negative and, not carrying the P pilus receptor, are not susceptible to colonization by P pili carrying strains. These people do not suffer from urinary infections mediated by the usual route (i.e., mucosal colonization followed by ascending invasion of the bladder). Such individuals may, however, become infected when the normal route is bypassed (e.g., by the use of an indwelling urinary catheter).

Other Invasive Strains

Strains that possess a sialic acid-containing capsular polysaccharide, called K1 antigen, cause invasive disease, such as septicemia and meningitis in infants. *E. coli* is also a common cause of septicemia in adults. Many patients with this manifestation acquire it as a consequence of infections of the urinary tract, often following manipulations such as urinary catheterizations or obstruction of urine flow. Cholecystitis is another common cause of *E. coli* bacteremia.

Principles of Diagnosis Using Clinical Specimens

Naturally occurring strains of *E. coli* are generally similar with respect to both their colonial morphology on agar plates and their shape under the microscope. *E. coli* can be distinguished from other enteric bacteria on the basis of biochemical and nutritional properties. Most strains of *E. coli* differ from some of the classic intestinal pathogens, such as *Salmonella* and *Shigella*, in that they ferment lactose. For this reason, lactose is included as the sole added sugar together with a pH indicator in agar media. Lactose fermenting colonies (presumptively those of *E. coli*) turn a distinctive color due to the production of acid.

With the help of an ingenious array of differential and selective media, it is usually simple to isolate *E. coli* from samples, such as feces, that contain a preponderance of other bacteria. These media and other special tests permit laboratories to narrow down the identification to the main genera of the Enterobacteriaceae.

Classifying *E. coli* into serological subgroups is not a task that most clinical laboratories are prepared to carry out. The serological reagents most readily *E. coli*, *General Biology* 269 available commercially are those directed against EPEC strains. EHEC strains of the serotype O157:H7 are nearly unique in their inability to ferment sorbitol. Nonfermenting colonies are detected on sorbitol containing MacConkey agar and confirmed with a sensitive and specific latex agglutination test. Molecular and fluorogenic typing methods useful for diagnostic purposes have been developed to detect toxins, pili, and other virulence factors.

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http://www.ecosal.org – *Escherichia coli* and *Salmonella*, Cellular and Molecular Biology

Ethanol

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Glossary

cellulose A homopolymer of glucose that occurs in crystalline bundles of individual chains.

furfural An aromatic aldehyde derived from pentose sugars during acid hydrolysis of hemicellulose.

hemicellulose A complex polymer of pentose and hexose sugars with acetyl and glucuronyl side chains.

homoethanol pathway Redox balanced fermentation of sugar to ethanol with near-stoichiometric yield.

lignocellulose The structural polymers that comprise the plant cell wall, including cellulose, hemicellulose, pectin, and lignin.

Abbreviations

ADH	alcohol dehydrogenase
AFEX	ammonia fiber explosion
CBP	consolidated bioprocessing
FBR	Fermentation Biochemistry Research Unit
LDH	lactate dehydrogenase
PDC	pyruvate decarboxlyase
PDH	pyruvate dehydrogenase
PFL	pyruvate formate lyase
SSF	simultaneous saccharification and fermentation
USDA	United States Department of Agriculture

Defining Statement

The economic viability of fuel ethanol production from biological materials has been increased by metabolic engineering. This includes engineering microbes that inherently produce ethanol to utilize a variety of substrates and engineering bacteria that inherently metabolize a variety of substrates to produce ethanol.

Introduction

In 2005, more than 300 billion gallons of petroleum were consumed in the USA, with two-thirds of this consumption being transportation fuels. Similarly, two-thirds of the crude oil consumed that year was supplied by foreign imports. Limited supply and increasing cost of petroleum, along with an enhanced awareness of the risks associated with the dependence on crude oil imports, have elevated the interest in alternative renewable energy sources. Despite this increased interest, renewable energy accounted for only 6% of the total energy consumption of USA in 2005. Among the various renewable energy sources, ethanol is emerging as the dominant alternate energy source due to its clean burning nature with no detectable CO or NOx emission. Henry Ford envisioned that ethanol would be the fuel powering his Model T automobile. Toward this objective, he even built an ethanol fermentation plant in 1908 in Atchison, Kansas. However, the availability of cheaper petroleum-based fuel during the 1940s completely replaced ethanol as the transportation fuel of choice. At present, ethanol is having a renaissance as an alternate fuel to petroleum for reasons listed above. While ethanol-blended gasoline contributes more energy than solar or wind sources, it still accounts for only 6% of the total renewable energy use. However, the production and use of ethanol as a transportation fuel is on the rise. In 2004, ethanol-based fuels provided the energy equivalent to 23 million gallons of gasoline. In 2006, 4.9 billion gallons of fuel ethanol were produced in the USA, an increase of nearly threefold from the 1.7 billion gallons produced in 2001.

Ethanol can be produced by both chemical and biological processes. Catalytic hydration of ethylene is a simple chemical method for production of ethanol, but this accounts for only a tiny fraction of the ethanol produced in the world today. Almost all of the industrially produced ethanol today comes from fermentation of sugars by one organism *Saccharomyces cerevisiae.*

Fermentation of sugars to ethanol predates mankind, and the fermentation of plant nectar rich in sugars to ethanol by yeast and bacteria is probably a process of natural evolution that benefited the microbes. Earliest record of human adaptation of ethanol fermentation dates back to the Neolithic period (8500–4000 BC) as evidenced by the presence of resinated wine in pottery from Hajji Firnz Tepe, in the Northern Zagros Mountains of present day Iran, that has been dated to about 5000 BC (http://www.museum.upenn.edu).

The main feedstock of industrial ethanol production is either sugar derived from sugar cane, sugar beets, or crops with high sugar content. In the USA, glucose from corn starch is the dominant feedstock for ethanol production. In all of these fermentations, a fraction of the solar energy stored in green plants via the conversion of carbon dioxide and water to biomass is utilized. About 90% of the dry weight of green plants is in the form of lignocellulose, making it the most abundant source of renewable energy worldwide. Lignocellulose is a complex material that consists mainly of the cell wall structural polymers cellulose, hemicellulose, pectin, and lignin (**Figure 1**). Cellulose, hemicellulose, and pectin are carbohydrate polymers that can be utilized by microbial biocatalysts for ethanol production after appropriate treatment. Lignin cannot be utilized for ethanol production since the average oxidation state of its carbon is significantly higher than that of ethanol. But lignin, which accounts for about 10–20% of total lignocellulosic biomass, can be used as a feedstock for production of various other specialty chemicals or can be burned as a fuel to supply the energy for ethanol production processes.

Brazil has successfully used sugar-derived ethanol as an automotive fuel for many years. Ethanol can also be produced from glucose derived from sugar crops or corn, or from lignocellulose. There continues to be a debate over the long-term implications of using food crops or crops grown on fertile soil for fuel production, and there is great interest in the utilization of hemicellulosic feedstocks.

Microbial production of ethanol invariably starts with sugar. Glucose is converted to pyruvate by the enzymes of glycolysis. This oxidation of glucose to two pyruvates is coupled with the production of energy, as ATP and two NADHs as reductant. Fermentation of sugars to ethanol differs from other types of microbial fermentations in that pyruvate is decarboxylated by a unique enzyme, pyruvate decarboxlyase (PDC; EC 4.1.1.1), to yield acetaldehyde (**Figure 2**). The acetaldehyde is then reduced to ethanol by alcohol dehydrogenase (ADH; EC 1.1.1.1) using the NADH generated during the conversion of glucose to pyruvate. The combination of the glycolysis reactions, PDC and ADH helps maintain the redox balance within the cell along with energy production to support continued growth of the microbe (eqns [1] and [2]). This conversion of sugars to ethanol is referred to as homoethanol fermentation pathway.

$$\begin{aligned}&\text{Glucose} + n\text{ADP} + n\text{Pi} + 2\text{NAD}^+\\&\quad\rightarrow 2\text{Pyruvate} + 2\text{NADH} + n\text{ATP}\end{aligned} \qquad [1]$$

$$2\text{Pyruvate} + 2\text{NADH} \rightarrow 2\text{Ethanol} + 2\text{CO}_2 + 2\text{NAD}^+ \qquad [2]$$

In this reaction the number of ATP produced per glucose is variable depending on the organism.

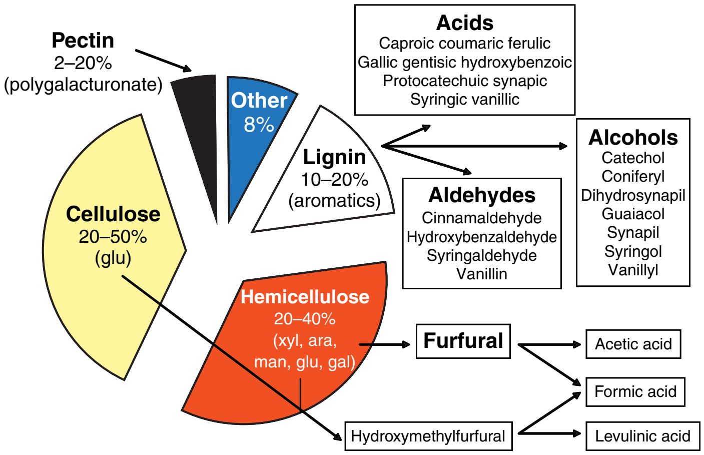
Pectin
2–20%
(polygalacturonate)
Other
8%
Lignin
10–20%
(aromatics)
Cellulose
20–50%
(glu)
Hemicellulose
20–40%
(xyl, ara,
man, glu, gal)
Acids
Caproic coumaric ferulic
Gallic gentisic hydroxybenzoic
Protocatechuic synapic
Syringic vanillic
Alcohols
Catechol
Coniferyl
Dihydrosynapil
Guaiacol
Synapil
Syringol
Vanillyl
Aldehydes
Cinnamaldehyde
Hydroxybenzaldehyde
Syringaldehyde
Vanillin
Furfural
Hydroxymethylfurfural
Acetic acid
Formic acid
Levulinic acid

Figure 1 Composition of lignocellulose and the toxins and inhibitors produced during dilute acid treatment. The average approximate lignocellulose composition is given as a percentage of total weight. ara, arabinose; gal, galactose; glu, glucose; man, mannose; xyl, xylose. Compounds enclosed within rectangular boxes are produced at varying levels during dilute acid hydrolysis of the lignocellulose.

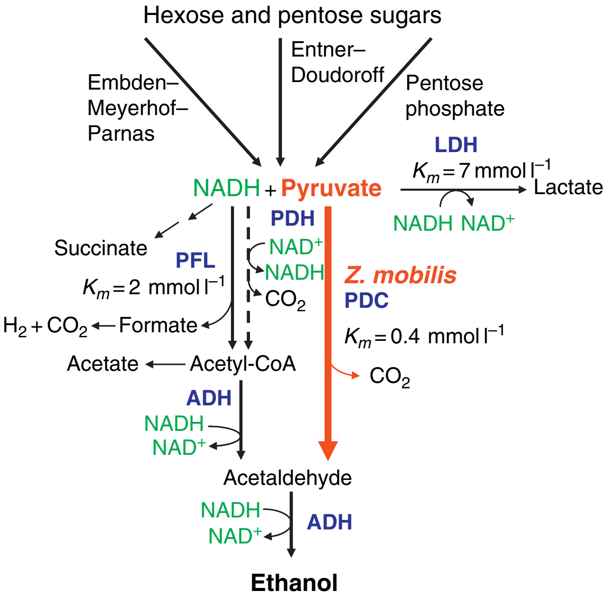

Figure 2 Metabolic pathways pertaining to ethanologenesis. PDH, which contains essential mutations for homoethanol production by *Escherichia coli* SE2378, is indicated with a dashed line. K_m values for pyruvate-utilizing reactions are shown. ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxlyase; PDH, pyruvate dehydrogenase; PFL, pyruvate-formate lyase.

Although PDC is common among yeast and other fungi, it is rare in bacteria. *Zymomonas mobilis, Acetobacter pasteurianus, Zymobacter palmae*, and *Sarcina ventriculi* are a few limited known examples of bacteria with the *pdc* gene encoding pyruvate decarboxylase. Among these bacteria, *Z. mobilis* and *Z. palmae* are the only bacteria that produce ethanol as the main fermentation product during normal anaerobic growth. Other bacteria are either aerobic or produce ethanol using PDC only during acid stress.

Production of ethanol from acetaldehyde by ADH is a common biological pathway. In most bacteria, acetaldehyde is formed from pyruvate formate lyase (PFL; EC 2.3.1.54)-derived acetyl-CoA. In contrast to nonoxidative decarboxylation of pyruvate to acetaldehyde by PDC, acetyl-CoA is produced by oxidative decarboxylation of pyruvate. The reductant generated during acetyl-CoA production is lost either as formate or H_2. Since, conversion of acetyl-CoA to acetaldehyde requires one NADH as reductant input, the overall conversion of pyruvate to ethanol requires two NADHs (**Figure 2**). Because glucose yields only enough NADH for the production of one ethanol, the remaining pyruvate is converted to organic acid, such as acetate. Due to this constraint for reductant, organisms that use acetyl-CoA as an intermediate in pyruvate metabolism can only convert, at most, half of the glucose to ethanol. Therefore, these organisms are not suitable microbial biocatalysts for homoethanol fermentation (eqns [1] and [3]).

$$\begin{aligned}\text{Glucose} &\rightarrow \text{2Pyruvate} + \text{2NADH}\\ &\rightarrow \text{Ethanol} + \text{2Formate} + \text{Acetate} \qquad [3]\end{aligned}$$

Based on the evaluation of ethanol-producing microbes, *S. cerevisiae* and *Z. mobilis* have emerged as the only viable naturally occurring microbial biocatalysts for fermentation of sugars to ethanol. Both *S. cerevisiae* and *Z. mobilis* ferment glucose completely to ethanol in a stoichiometric manner. Historically, yeast has served as the major ethanologenic microbial biocatalyst and is used exclusively by the ethanol industry to ferment sugar (from sugar cane, sugar beet, etc.) and glucose derived from various starch (such as corn starch) to ethanol. However, it lacks the inherent ability to ferment the pentose sugars that account for about 20–40% of lignocellulosic biomass. Since both *S. cerevisiae* and *Z. mobilis* lack the ability to ferment this resource, a significant portion of the fermentable sugar in biomass is left behind, reducing the overall ethanol yield during fermentation of biomass-derived sugars. Recent progress in metabolic engineering of these microbes to expand their substrate range has been covered in several excellent reviews (see the section ‘Further reading’). This article focuses on metabolic engineering of nontraditional ethanologenic bacteria, such as *Escherichia coli* and *Klebsiella oxytoca*, with the substrate range to ferment all of the sugars found in a wide range of lignocellulosic materials. In addition, other challenges to cost-effective ethanol production, such as the inhibitory properties of hemicellulose acid-hydrolysate and design of growth media are discussed.

Engineering *E. coli* for Ethanol Production

While *Saccharomyces* and *Z. mobilis* natively express the homoethanol pathway, both lack the ability to metabolize the pentose sugars that are abundant in hemicellulose. Alternatively, *E. coli* lacks the homoethanol pathway but is able to utilize a wide variety of substrates, such as hexose and pentose sugars and the uronic acid found in pectin. Other advantages of using *E. coli* for ethanol production are the extensive understanding of its physiology and metabolism and ease of genetic manipulation. Therefore, *E. coli* was an obvious choice for metabolic engineering toward homoethanol production.

KO11: Versatile Microbial Biocatalyst for Biomass Conversion to Ethanol

The *Z. mobilis* genes encoding PDC and ADH (homoethanol pathway, PET operon) were cloned into a plasmid and expressed from the PFL promoter in *E. coli*. The *Z. mobilis* PDC has a significantly higher affinity for pyruvate (K_m of 0.4 mmol l^{-1}) than the native PFL (K_m of 2 mmol l^{-1}) or lactate dehydrogenase (LDH; EC 1.1.1.28) (K_m, 7 mmol l^{-1}) allowing for effective competition for the available pyruvate pool (**Figure 2**). With the goal of finding the best *E. coli* host for further strain development, eight wild-type *E. coli* strains were tested for ethanol tolerance and productivity in medium rich in 100 g l^{-1} glucose. The *E. coli* strains ATCC 9637 (W), ATCC 8739 (Crooks), and ATCC 14948 (K-12 W3100) showed the maximum ethanol tolerance, and ATCC 11303 (*E. coli* B), ATCC 11775, and ATCC 15224 demonstrated the highest PDC activity from the plasmid-borne homoethanol pathway genes.

To eliminate plasmid dependence, the PET genes were chromosomally integrated into the *pfl* locus along with a chloramphenicol resistance gene into wild-type *E. coli* strain W. To increase ethanol production, spontaneous mutants with increased ADH activity and resistance to 600 mg l^{-1} of chloramphenicol were selected. Undesirable carbon loss through nonethanol *E. coli* fermentation pathways was decreased by deletion of the succinate-producing *frd* gene. Production of ethanol by the resulting strain, KO11, from 100 g l^{-1} glucose or 80 g l^{-1} xylose in rich medium exceeded the theoretical yield due to the presence of complex nutrients in the growth medium. Strain KO11 was initially reported as a derivative of *E. coli* B, but further comparative genome sequence analysis of several wild-type *E. coli* strains and KO11 revealed that the genome of strain KO11 is most similar to that of *E. coli* W and should be considered as a derivative of *E. coli* W (ATCC 9637).

Although the PDC produced by strain KO11 had a significant advantage over the other two enzymes that metabolize pyruvate, growing cultures of KO11 produced acetate during growth, especially during growth with xylose. This indicates that PFL activity was also contributing to pyruvate metabolism in this organism. Since the conversion of acetyl-CoA to acetate yields one additional ATP, PFL-mediated production of acetate and ethanol apparently contributed to higher growth rate and cell yield, which are essential components for an increased volumetric productivity of the microbial biocatalyst. With a decrease in the growth rate as the culture reaches the stationary phase of growth, wild-type *E. coli* switches its fermentation product to lactate, a reaction that oxidizes NADH without producing ATP. Although the PDC-mediated ethanol pathway and LDH both oxidize NADH, the higher affinity of PDC for pyruvate effectively channels the pyruvate to ethanol. Because these physiological advantages negate the need to delete PFL and LDH as PDC-competing reactions, PFL and LDH were not removed from strain KO11.

In the 20 years since the ethanologenic *E. coli* strain KO11 was described, it has been used to catalyze the conversion of many types of biomaterials to ethanol (**Table 1**). These substrates include, but are not limited to, sugars derived from sugar cane bagasse, corn cobs, hulls and AFEX-pretreated fibers, pectin-rich beet pulp, willow, sweet whey, rice hulls, brewery waste, cotton gin waste, *Pinus* sp. hydrolysate, housing wood waste, and orange peels.

Table 1 Biomass conversion by ethanologenic biocatalysts

Organism	*Biomass*
Escherichia coli KO11	Sugar cane bagasse
	Sugar cane molasses
	Rice hulls
	Beet pulp
	Corn hulls and fibers
	Corn hulls
	Orange peel
	Sweet whey
	Brewery wastewater
	Pinus sp. (softwood)
	Willow (hardwood)
	Waste housing wood
E. coli FBR	Wheat straw
	Corn ‘quick fiber’
	Corn fiber
Klebsiella oxytoca P2	Crystalline cellulose
	Mixed waste office paper
	Sugar cane molasses
	Sugar cane bagasse
Zymomonas mobilis ZM4 (pZB5)	Oat hull
	Wheat stillage

As desired for a successful industrial biocatalyst, KO11 was shown to be robust to soil contamination, moderate temperatures, and pH values. Industrially competent ethanologenic biocatalysts need to retain ethanologenicity without dependence on antibiotics to retain the introduced genes and their expression. Strain KO11 has been reported to maintain stable ethanol yields for up to 27 days in fluidized bed and continuous stirred tank reactors without inclusion of chloramphenicol.

The process cost of bioethanol production is dependent upon a multitude of factors. From the fermentation point of view, the critical external factor, besides the microbial biocatalyst, is the amount of nutritional supplements required by the microbe for optimum activity. While KO11 has demonstrated the desired ability to produce ethanol from a wide variety of biomaterials, supplementation of the medium with complex nutrients did increase the overall ethanol productivity (**Table 2**). In rich medium, KO11 converted 100 g l^{-1} glucose to 45 g l^{-1} ethanol within 72 h, but produced only 30 g l^{-1} ethanol even after 96 h in mineral salts medium. This positive effect of nutritional supplements on ethanol productivity was attributed to higher efficiency of PDC in converting pyruvate to acetaldehyde at the expense of decreased production of acetyl-CoA that is needed for biosynthesis. Additionally, KO11 ADH activity *in vivo*

Table 2 Comparison of ethanologenesis

Organism	*Sugar*[a] ($g\,l^{-1}$)	*Medium*	*Ethanol*[b] ($g\,l^{-1}$)	*Yield* ($g\,g^{-1}$)	*Reference*
Escherichia coli KO11	90, xyl	LB	43.2	0.48	1
E. coli KO11	90, xyl	Min	26.9	0.30	1
E. coli LY01	90, xyl	LB	42.4	0.47	2
E. coli LY168	90, xyl	Min	45.5	0.51	1
E. coli LY168	90, xyl	LB	45.3	0.50	1
E. coli FBR5 (pLOI297)	95, xyl	LB	41.5	0.44	3
E. coli SE2378	50, xyl	LB	20.5	0.41	4
Klebsiella oxytoca P2	100, glc	LB	45.2	0.49	5
Zymomonas mobilis ZM4 (pZB5)	50, glc; 50, xyl	YE	50.0	0.50	6
Z. mobilis CP4 (pZB5)	8, glc; 80, xyl	YE	36.6	0.48	7
Saccharomyces cerevisiae RE700A (pKDR)	50, xyl	YE	23.0	0.46	8
S. cerevisiae RWB217	20, xyl	Synth	8.7	0.44	9

[a] Initial sugar concentration.

[b] Final reported concentration in the medium.

glc, glucose; LB, Luria Broth; Min, mineral salts medium + 1 mmol l^{-1} betaine; Synth, minerals supplemented with a mixture of vitamins; YE, medium containing yeast extract; xyl, xylose.

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may not have matched the efficiency of the PDC activity, leading to a higher than expected NADH pool size. High NADH levels inhibit the activity of citrate synthase, the first committed enzyme toward synthesis of 2-oxoglutarate, a precursor to glutamate, an important compound for osmotic tolerance and amino acid biosynthesis. Expression of an NADH-insensitive citrate synthase from *Bacillus* increased the growth of strain KO11 and ethanol production by about 75%, supporting the conclusion that citrate synthase inhibition by NADH contributed to the lower productivity of KO11. Supplementation of the growth medium with an osmoprotectant, such as betaine, also helped overcome the need for complex nutrients to enhance the ethanol productivity of strain KO11, indicating the efficiency of this microbial biocatalyst in converting sugars to ethanol.

All ethanologenic microbes produced ethyl acetate as a minor byproduct of ethanol fermentation. Since there are multiple enzymes that catalyze the production of ethyl acetate, a *Pseudomonas putida* esterase (*estZ*) was introduced into KO11 to reduce the concentration by hydrolyzing the ethyl acetate to ethanol and acetate. In KO11 fermentations, the ethyl acetate levels were reduced to less than 20 mg l^{-1} by the esterase activity, similar to the amount produced by yeast.

LY01: Increased Ethanol Tolerance

Despite a comparable rate of ethanol production to yeast strains, the ethanol tolerance of KO11 is lower than commercially employed yeast strains: 35 g l^{-1} ethanol inhibited the growth of KO11 even in rich medium compared to the tolerance level of at least 200 g l^{-1} ethanol for *S. cerevisiae.* In an effort to increase the ethanol tolerance of ethanologenic *E. coli*, KO11 was evolved for higher ethanol tolerance and productivity for 3 months in media with increasing concentrations of ethanol. This metabolic evolution consisted of alternating periods of selection for increased ethanol productivity and increased ethanol tolerance. The resulting strain, LY01, demonstrated higher than 80% survival to short-term exposure to $100\,g\,l^{-1}$ ethanol and was able to grow in the presence of $50\,g\,l^{-1}$ ethanol.

With the goal of identifying metabolic changes that contributed to increased ethanol tolerance, total mRNA pools of KO11 and LY01 were compared during growth in rich medium with $100\,g\,l^{-1}$ glucose or xylose and 0, 10, or $20\,g\,l^{-1}$ ethanol. This analysis identified 205 genes with significantly different expression in KO11 and LY01; for 49 of these genes, the expression difference was higher than twofold. The differentially expressed genes belong to a variety of functional groups, including cell processes, cell structure, amino acid biosynthesis, central intermediary metabolism, energy metabolism, and stress response. This analysis suggested three major physiological differences between KO11 and LY01 that lead to higher ethanol tolerance of *E. coli*: increased glycine degradation, increased uptake of protective osmolytes and betaine synthesis, and lack of function of the transcription regulator, FNR protein. When functional, FNR controls the expression of fermentation and anaerobic respiration genes. As described above, suboptimal pyruvate distribution contributes to limited growth of KO11 in minimal medium. Therefore, it is interesting to note that glycine metabolism and the FNR regulon, which both impact pyruvate availability and distribution, are significantly altered in strain LY01. As expected, the increase in ethanol tolerance of LY01 is a result of a combination of multiple physiological factors.

Ethanologenic *E. coli* Strain LY168: Adapted for Mineral Salts Medium

Due to the suboptimal distribution of pyruvate for biosynthesis and the osmotic stress of high initial sugar concentrations during ethanol fermentation, the ethanologenic *E. coli* strains described thus far required supplementation with complex nutrients for complete fermentation of sugars, such as $90\,g\,l^{-1}$ xylose. Since nutritional supplements increase the process cost of ethanol production, a new strain of ethanologenic *E. coli* was constructed (strain LY168) that can optimally grow and ferment high concentrations of sugar in mineral salts medium with only betaine added as an osmoprotectant.

The key difference between strain LY168 and strain KO11 is in the way that expression of the homoethanol pathway is regulated. In strain KO11, the *Z. mobilis pdc* and *adh* genes are regulated by the *pfl* promoter with multiple layers of transcriptional control. In strain LY168, the genes encoding the homoethanol pathway are integrated within the gene encoding 23S ribosomal RNA subunit *rrlE*, concurrent with the direction of transcription. Since rRNA synthesis is growth rate controlled, complex regulation of the two promoters results in high expression of ribosomal RNA at high growth rates and basal expression during stationary phase and low growth rates. Therefore, in retrospect, a ribosomal RNA promoter is an ideal choice for expression of the *Z. mobilis* homoethanol pathway. Further, metabolic evolution and mutations leading to strain LY168 optimized the level of expression of the PET operon that supported growth of the ethanologen in mineral salts medium.

As desired, strain LY168 produced $45.5\,g\,l^{-1}$ ethanol from $90\,g\,l^{-1}$ xylose in mineral salts medium with betaine alone as an osmoprotectant within 48 h (**Table 2**). This yield of 0.5 g ethanol per gram of xylose is very close to the maximum theoretical yield of 0.51. Even more important is the fact that the volumetric and specific productivities of strain LY168 were independent of the presence or absence of additional nutrients in the mineral salts medium (**Table 2**). Strain LY168 met the need for a biocatalyst that rapidly and efficiently converts sugars to ethanol without depending on expensive nutritional supplements.

FBR Series: Sustained Ethanologenicity

The Fermentation Biochemistry Research Unit at USDA has also designed a series of ethanologenic *E. coli* K12 derivatives with the goal of maximizing strain stability. As with KO11 and LY168, these strains express the *Z. mobilis* homoethanol pathway. However, the homoethanol pathway is maintained on a plasmid (pLOI297) in these strains instead of the chromosomal integration in strains KO11 and LY168. Since the FBR strains lack both LDH and PFL, growth is dependent on expression of the homoethanol pathway for NAD^+ regeneration and redox balance under anaerobic conditions. This need for the homoethanol pathway for anaerobic growth negates the need for antibiotics in the medium for stable plasmid maintenance.

FBR5, the most recent strain of this series, produced ethanol from wheat straw, corn fibers, and corn 'quick fiber', a modified corn grind that allows recovery of fiber fractions prior to fermentation (**Table 1**). Consistent with the design goal, FBR5 maintained stable ethanol yields during 26 days of continuous culture on glucose or xylose. However, like KO11, these strains are dependent on rich medium for optimum rate of growth and fermentation. Additionally, the final ethanol titer and yield in rich media are lower than those of LY168 in minimal medium (**Table 2**).

SE2378: Nonrecombinant Homoethanol Production

The ethanologenic *E. coli* strains described thus far have utilized the *Z. mobilis* pathway for homoethanol production. However, a recently isolated mutant *E. coli* strain demonstrated a new metabolic pathway that can lead to balanced production of ethanol without foreign genes.

During anaerobic growth, PFL and LDH serve as the major routes for NAD$^+$ regeneration in *E. coli* (**Figure 2**), and mutants that lack the genes encoding these two proteins are incapable of anaerobic growth. This physiological property was used to stabilize the ethanologenic plasmid in the FBR strains described above. Although *E. coli* produced pyruvate dehydrogenase (PDH) under both aerobic and anaerobic growth conditions, activity of this enzyme is inhibited by the high NADH pool levels of an anaerobic cell. A mutation in the genes encoding the PDH complex that reduced the level of enzyme inhibition by NADH supported the activity of PDH under anaerobic growth condition. In such a mutant, strain SE2378, PDH provided an alternate route of pyruvate metabolism that led to the production of acetyl-CoA and NADH. With this additional NADH (2 NADHs per acetyl-CoA), balanced production of one ethanol per pyruvate was achieved (**Figure 2**). Although the maximum specific ethanol productivity of the strain SE2378, $2.24\,\mathrm{g\,h^{-1}\,(g\ cell)^{-1}}$ with $50\,\mathrm{g\,l^{-1}}$ xylose, was comparable to recombinant ethanologenic *E. coli* KO11, strain SE2378 still requires further metabolic engineering to increase the growth rate and volumetric ethanol productivity to levels that are comparable to those of *S. cerevisiae* and *E. coli* LY168.

This glycolysis- and PDH-based conversion of one mole of glucose to two moles of ethanol is a new pathway that was not previously known in nature. Since all facultative bacteria produce PDH during aerobic growth, this new homoethanol pathway provides an alternate to the PDC/ADH pathway toward engineering an expanded list of microbial biocatalysts for ethanol production from lignocellulose.

Other Engineered Ethanologenic Microbial Biocatalysts

Klebsiella oxytoca

One of the motivational factors for using *E. coli* as an ethanologen is the fact that it utilizes a wide variety of biomass-derived sugars. *K. oxytoca*, in addition to metabolizing monomeric sugars, also has the native ability to transport and metabolize the cellulose subunits cellobiose and cellotriose. Engineering of *K. oxytoca* for ethanol production, resulting in strain P2, paralleled recombinant expression and chromosomal integration of the *Z. mobilis* PET operon in *E. coli* by the Ingram Lab. Production of ethanol from a variety of substrates by *K. oxytoca* P2 is presented in **Table 1**. Another difference between *K. oxytoca* and *E. coli* is the ability of *K. oxytoca* to use urea as sole nitrogen source. The use of urea reduces media acidification and therefore reduces the process cost. In mineral salts urea medium, *K. oxytoca* strain BW21 produced more than $40\,\mathrm{g\,l^{-1}}$ ethanol from $90\,\mathrm{g\,l^{-1}}$ glucose within 48 h.

Gram-Positive Biocatalysts

The robustness of Gram-positive organisms makes them industrially appealing. While the *Z. mobilis* pathway has been successfully expressed in *Corynebacterium glutamicum*, initial engineering attempts with lactic acid bacteria and *Bacillus* were only limitedly successful.

Ethanol Production by *Z. mobilis*

The ethanologenic potential of microbial biocatalyst *Z. mobilis* was first reported over 25 years ago. Like *S. cerevisiae*, *Z. mobilis* contains PDC and is capable of glucose fermentation to ethanol through the homoethanol pathway at a rate and volumetric productivity that exceeds that of yeast. Unfortunately, *Z. mobilis* also shares a shortcoming with yeast: it lacks the inherent ability to utilize the pentose sugars that are abundant in biomass. Recent whole-genome sequencing confirmed the lack of key pentose–phosphate pathway enzymes in this bacterium.

The presence of a facilitated glucose diffusion system in *Z. mobilis* allows rapid equilibration between internal and external glucose concentrations. However, *Z. mobilis* is unusual in that it lacks 6-phosphofructokinase and therefore uses the Entner–Doudoroff pathway to metabolize glucose instead of the Embden–Meyerhoff–Parnas pathway. In addition to glucose utilization, *Z. mobilis* is also capable of producing ethanol from fructose, sucrose, desalted molasses, aspen, corn, wheat, natural rubber waste, Jerusalem artichoke juice, and cassava.

Much of the fermentation research has focused on immobilization of *Z. mobilis* cells on glass beads, to increase biocatalyst loading and therefore ethanol productivity. *Z. mobilis* productivity has also been improved by metabolic evolution and mutagenesis. Metabolic evolution of strain CP4 during 18 months of serial transfers enabled the production of $95\,\mathrm{g\,l^{-1}}$ ethanol in just 17.4 h instead of the 31.8 h required by the original strain. *Z. mobilis* strains with increased ethanol tolerance, improved growth on mannitol, increased osmotic tolerance, and increased thermotolerance were isolated after mutagenesis.

However, metabolic engineering and mutagenesis were unable to compensate for the lack of pentose utilization genes, and there were many attempts to engineer pentose utilization ability by the incorporation of foreign genes into *Z. mobilis*, with limited initial success. However, by using *Z. mobilis* promoters to regulate the expression of *E. coli* genes encoding xylose isomerase, xylulokinase, transketolase, and transaldolase, a xylose-fermenting

Z. mobilis CP4 (pZB5) was constructed. This strain fermented xylose and produced ethanol at 86% of the theoretical yield (**Table 2**). Similarly, the use of *Z. mobilis* promoters to regulate *E. coli* arabinose metabolic genes resulted in the utilization of arabinose as the sole carbon source and production of ethanol at 98% of the theoretical yield. Recombinant strain ZM4 (pZB5) produced $50\,\mathrm{g\,l^{-1}}$ ethanol from a mixture of $50\,\mathrm{g\,l^{-1}}$ glucose and $50\,\mathrm{g\,l^{-1}}$ xylose, a yield of 0.50 g ethanol per gram of sugar (**Table 2**). Biomass utilization by strain ZM4 (pZB5) is detailed in **Table 1**. Immobilized *Z. mobilis* 31821 (pZB5) has also been shown to produce ethanol from various biomaterials including cedar, newspaper, and rice straw after treatment. *Z. mobilis* 8b, which contains chromosomally integrated pZB5, produced ethanol at 85% yield from acid-treated corn stover.

Challenges for Cost-Effective Ethanol Production

Fungal Cellulase Supplementation

The insoluble crystalline structure of cellulose makes it relatively inaccessible for microbial digestion. Therefore, the cellulose must be treated by either chemical or physical means for appreciable ethanol production.

The fungal cellulases traditionally used for cellulose hydrolysis are costly and have a lower catalytic rate than other glycosidases. This process is further complicated by the fact that cellulase activity is inhibited by the hydrolysis products glucose and cellobiose. To combat this product inhibition, Gulf Oil Company developed the simultaneous saccharification and fermentation (SSF) process in 1976. As the name implies, this process combines cellulose saccharification and *Saccharomyces*-mediated fermentation of the resultant glucose in a single reaction vessel.

Recent attempts to reduce the cost of fungal cellulase have focused on engineering biocatalysts to produce the required cellulase enzymes themselves, a process referred to as consolidated bioprocessing (CBP). A strain of *S. cerevisiae* engineered to express the *Trichoderma reesei* endoglucanase II and cellobiohydrolyase II and *Aspergillus aculeatus* β-glucosidase 1 produced ethanol directly from amorphous cellulose at 88.5% of the theoretical yield without fungal cellulase supplementation. Work with recombinant *E. coli* and *K. oxytoca* has focused on engineered expression of the CelZ and CelY endoglucanases of *Erwinia chrysanthemi*. These enzymes work synergistically to degrade amorphous cellulose and carboxymethyl cellulose. Preliminary work showed that effective reduction of the cellulase demand required high expression and secretion of CelZ and CelY by the microbial biocatalysts. The use of a surrogate *Z. mobilis* promoter for *celZ* and simultaneous expression of the *E. chrysanthemi out* secretion system resulted in the expression of active glycan hydrolase as approximately 5% of the total cellular protein by both *E. coli* and *K. oxytoca*. Additionally, these enzymes enabled *K. oxytoca* to convert amorphous cellulose to ethanol at 58–76% of the theoretical yield without the addition of any supplemental enzymes.

As described above, cellobiose, the product of cellulose digestion, inhibits the cellulose digestion enzymes. Therefore, biocatalysts that can prevent cellobiose accumulation via metabolic consumption are industrially appealing. The ability to metabolize cellobiose is widespread in prokaryotes, including *K. oxytoca*. Functional expression of the *K. oxytoca casAB* cellobiose utilization operon in *E. coli* KO11 resulted in ethanol production from cellobiose at greater than 90% of the theoretical yield, and this derivative also fermented mixed-waste office paper to ethanol with the aid of commercial cellulase. Recombinant expression of the *Ruminococcus albus* β-glucosidase gene enabled *Z. palmae* to produce ethanol from cellobiose at 95% of the theoretical yield.

Several physical and chemical cellulose treatment methods to make the crystalline cellulose accessible to the hydrolytic enzymes, coupled with engineering of microbial biocatalysts with cellulolytic capability, are being developed for SSF of cellulose toward addressing the need for ethanol production from cellulose in a cost-effective manner.

Hemicellulose Hydrolysate-Containing Inhibitors

As with cellulose, hemicellulose requires depolymerization of its soluble components prior to bacterial utilization. The various methods of hemicellulose hydrolysis for ethanol production have been recently reviewed, and this article focuses only on hydrolysis with dilute mineral acid at modest temperatures. This method, which yields xylose-rich syrup, also produces significant amounts of various compounds that inhibit growth of the microbial biocatalysts and limit ethanol production (**Figure 1**).

One of the most thoroughly studied inhibitors in hemicellulose hydrolysate is furfural, an aromatic aldehyde derived from pentose sugars. Furfural concentrations in hemicellulose hydrolysates range from 1 to $4\,\mathrm{g\,l^{-1}}$. Furfural concentrations as low as $2.4\,\mathrm{g\,l^{-1}}$ can inhibit *E. coli* growth in rich medium, and the sensitivity is increased in mineral salts medium. There are other hemicellulose-derived aldehydes that are more toxic than furfural on a weight basis, such as 4-hydroxybenzaldehyde and syringaldehyde, but furfural is of particular interest because it enhances the inhibitory effects of other toxins on *E. coli*. Ethanologenic biocatalysts *E. coli* KO11 and LY01 and *K. oxytoca* P2 have the ability to convert furfural to furfuryl alcohol via an unidentified NAD(P)H-dependent alcohol–aldehyde oxidoreductase.

As described above, LY01 was derived from KO11 following selection for spontaneous mutants with increased ethanol tolerance. Surprisingly, LY01 also has increased furfural tolerance, as indicated by the ability to grow, although at a reduced rate, in the presence of $3\,\mathrm{g\,l^{-1}}$ furfural, a concentration that completely inhibits KO11 growth.

Alcohol, aldehyde, and acid components of hemicellulose hydrolysate impact *E. coli* LY01 in a variety of ways. For the compounds tested, the toxicity was directly related to the hydrophobicity of the molecule. All of the aldehyde compounds inhibited growth of LY01, but only furfural reduced ethanol production. The toxicity of alcohols, such as catechol and hydroquinone, was lower than that of aldehydes and acids, and the inhibitory effect of these alcohols on ethanol production appeared to be a secondary effect of growth inhibition. Aliphatic and mononuclear organic acids inhibited growth and ethanol production by probably disturbing ion gradients and increasing the intracellular anion concentration. This is especially true for acetic acid, that is present in the hemicellulose hydrolysate at a concentration as high as $10\,\mathrm{g\,l^{-1}}$, where $9\,\mathrm{g\,l^{-1}}$ was sufficient for growth inhibition of *E. coli* KO11 in rich medium.

Like *E. coli*, *S. cerevisiae* is inhibited by hemicellulose-derived furans, acids, and phenolic compounds, with strain-dependent variation in the degree of sensitivity. The inhibitor tolerance of *S. cerevisiae* strains was increased via overexpression of alcohol dehydrogenase ADH6p for increased furfural reduction or phenylacrylic acid decarboxylase PAD1 for increased consumption of phenolic derivatives. Metabolic evolution in synthetic media with increasing concentrations of inhibitors was used to develop *S. cerevisiae* strains with increased inhibitor tolerance. Additionally, several *S. cerevisiae* strains were found to successfully ferment hemicellulose hydrolysate without detoxification.

Just as there are various methods for generating hemicellulose hydrolysate, there also exist various methods for reducing its toxicity. One common method, termed ‘overliming’, involves adjustment of the hydrolysate pH to 9–10 by adding calcium hydroxide. Overliming of sugar cane bagasse acid-hydrolysate enabled the conversion of $95\,\mathrm{g\,l^{-1}}$ sugar to $33\,\mathrm{g\,l^{-1}}$ ethanol by *E. coli* LY01, compared to the production of $<1\,\mathrm{g\,l^{-1}}$ ethanol from the same hydrolysate that had only been adjusted to pH 6.5–6.7. Comparison of overliming corn stover hydrolysate to pH 9–11 showed that pH 10 had the best results for fermentation by *Z. mobilis* 8b. While overliming to pH 11 produced the most fermentable hydrolysate, loss of xylose was substantial at this pH. Alternative methods of toxicity reduction include ion exchange resins, adsorptive membranes, and charcoal treatment.

Growth Media Engineering

In addition to engineering strains to require less nutritional supplementation for rapid, efficient attainment of high ethanol concentrations, progress has also been made in engineering simpler, cheaper growth media for cultivation of microbial biocatalysts.

For processes that still require complex nutrient supplementation, on-site preparation of crude autolysate from spent yeast allows synergy between grain-based and lignocellulosic processes.

Osmolyte Stress

To produce ethanol at the desired high concentrations, fermentation must start with high levels of substrate sugars. Sugars at these high concentrations generate osmotic stress that is intensified by growing the microbial biocatalysts in mineral salts medium. Protective osmolytes, such as glutamate, betaine, trehalose, and proline, help bacteria maintain the appropriate cell volume and turgor despite changes in extracellular osmolality. As described above, lower ethanol productivity of *E. coli* KO11 grown in minimal medium compared to that in rich medium was attributed to lower levels of osmoprotective glutamate produced by these cultures. Intracellular levels of other osmolytes were also low during anaerobic growth of *E. coli* relative to aerobic growth in the same medium. Increasing the level of expression of trehalose synthesis genes *otsBA* alleviated the osmotic stress of *E. coli* induced by high sugar concentrations. Additionally, a combination of increased osmolyte synthesis and supplementation with betaine increased the *E. coli* tolerance to high sugar concentrations more than trehalose synthesis or betaine supplementation alone.

Conclusion

Metabolic engineering has proved invaluable to economically viable production of ethanol as an alternative fuel. Microbial biocatalysts that inherently produce ethanol have been improved by the introduction of additional sugar utilization pathways, and others that inherently utilize a wide spectrum of sugars have been improved by the introduction of the homoethanol pathway. Metabolic evolution has further improved the performance of these microbial biocatalysts in cost-effective growth media. Metabolic engineering has also contributed to a reduction in demand for supplemental cellulase enzymes for the conversion of lignocellulosic biomass to ethanol. There are frequent reports on the identification of new bacteria with high ethanol tolerance and/or productivity, and additional studies and engineering of these microbial biocatalysts are expected to yield continued improvement and sustainability of ethanol production.

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Relevant Website

http://www.museum.upenn.edu – Penn Museum, University of Pennsylvania

Evolution, Theory and Experiments with Microorganisms

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Defining Statement
Review of Evolutionary Theory
Experimental Tests of Fundamental Principles
Genetic and Physiological Bases of Fitness
Genetic Variation within Populations
Coevolution of Interacting Genomes and Species
Evolution of New Metabolic Functions
Evolution of Genetic Systems
Further Reading

Glossary

adaptation A feature of an organism that enhances its reproductive success and that evolved by natural selection.

evolution Change in the genetic properties of populations and species over generations, which requires the origin of variation (by mutation or mixis) as well as the subsequent spread or extinction of variants (by natural selection and genetic drift).

fitness Average reproductive success of a genotype in a particular environment, usually expressed relative to another genotype.

genetic drift Changes in gene frequency caused by the random sampling of genes during transmission across generations (rather than by natural selection).

mixis Production of a new genotype by recombination of genes from two sources.

natural selection Changes in gene frequency caused by specific detrimental or beneficial effects of those genes.

population Group of individuals belonging to the same species and living in close proximity, so that individuals may potentially recombine their genes, compete for limiting resources, or otherwise interact.

Abbreviations

GASP	Growth Advantage in Stationary Phase
MOI	multiplicity of infection

Defining Statement

Evolution in action can be studied by experiments in the laboratory using bacteria and other microorganisms with suitably rapid generation. These experiments have confirmed the main principles of modern evolutionary theory, while also providing new insights into the genetics, physiology, and ecology of microorganisms.

Review of Evolutionary Theory

Evolutionary theory seeks to explain observable patterns of biological diversity in terms of a few fundamental evolutionary processes. These processes are presumed not only to have operated in the past, but also to continue to operate today. Thus, they can be studied experimentally in the laboratory. Before discussing a broad range of experiments that have used microorganisms to examine evolutionary processes, the major elements of evolutionary theory will be reviewed.

Evolutionary Patterns

The three most conspicuous products of organic evolution are (1) the wealth of genetic variation that exists within almost every species; (2) the divergence of populations and species from one another and from their common ancestors; and (3) the manifest adaptation, or fit, of organisms to the environments in which they live.

Genetic variation

The existence of extensive genetic variation within species has been demonstrated by a variety of means. Variation in certain traits, such as seed shape in pea plants and blood type in humans, can be shown to have a genetic basis by careful examination of pedigrees. For many other traits, such as milk production in cows or body weight in humans, quantitative genetic analyses are required to partition the phenotypic variation that is due to genetic versus environmental influences. Biochemical and molecular techniques have also revealed extensive variation in DNA sequences and the proteins they encode.

Divergence and speciation

All biological species differ from one another in some respects. It is generally possible to arrange species hierarchically, depending on the extent and nature of their similarities and differences. This hierarchy is reflected in the classification scheme of Linnaeus (species, genus, family, and so on). This hierarchical arrangement also suggests a sort of 'tree of life' in which the degree of taxonomic relatedness between any two species reflects descent with modification from some common ancestor in the more or less distant past. Investigating the origins of groups and their relationships requires an historical approach, which is not amenable to direct experimentation. Even so, historically based hypotheses can be tested by phylogenetic and comparative methods, which utilize data on the distribution of traits across various groups and environments, sometimes supplemented with information from the fossil record.

The extent of evolutionary divergence that is necessary for two groups of organisms to be regarded as distinct species is embodied in the biological species concept, according to which 'species are groups of actually or potentially interbreeding populations, which are reproductively isolated from other such groups' (E. Mayr, in 1942). Speciation thus refers to the historical process by which groups of organisms become so different from one another that they no longer can interbreed. However, many organisms (including most microorganisms) reproduce primarily or exclusively asexually, and the preceding definition is not applicable. For such organisms, the extent of evolutionary divergence that corresponds to distinct species is somewhat arbitrary and often more a matter of convenience than of scientific principle.

Adaptation

Many phenotypic features of organisms often exhibit an exquisite match to their environments. For example, the bacteria that live in hot springs have special physiological and biochemical properties that allow them to survive and grow at very high temperatures, which would kill most other bacteria; often these thermophiles cannot grow at all under the lower temperatures where most other bacteria thrive. However, organisms are also generally not perfectly adapted to their environments. Evidence for the imperfection of organisms can be seen when species go extinct, usually as a consequence of some change in the environment to which they cannot quickly adapt.

Evolutionary Processes

Biological evolution occurs whenever the genetic composition of a population or species changes over a period of generations. Four basic processes contribute to such change: mutation, mixis, natural selection, and genetic drift. Selection and drift will not produce evolutionary changes, however, unless there exists genetic variation among individual organisms.

Sources of genetic variation

Genetic variation among individuals is generated by two distinct processes, mutation and mixis. In terms of evolutionary theory, these processes are usually distinguished as follows: mutation refers to a change at a single gene locus from one allelic state to another (e.g., $abcd \rightarrow Abcd$, where each letter indicates a locus), whereas mixis refers to the production of some new multilocus genotype by the recombination of two different genotypes (e.g., $abcd + ABCD \rightarrow aBcD$).

There are many different types of mutations, including point mutations, rearrangements, and transposition of mobile elements from one site in the genome to another. Some mutations cause major changes in an organism's phenotype; for example, a bacterium may become resistant to attack by a virus (bacteriophage) as a result of a mutation that alters a receptor on the cell surface. Other mutations have little or even no effect on an organism's phenotype: many point mutations have absolutely no effect on amino acid sequence (and hence protein structure and function) because of the redundancy in the genetic code.

Any number of factors may affect mutation rates, including both environmental agents (e.g., UV irradiation) and the organism's own genetic constitution (e.g., defective DNA repair genes). Evolutionary theory makes no assumptions about the rates of mutations or their biophysical bases, with one exception: mutations are assumed to occur randomly, that is, irrespective of their beneficial or harmful effects on the organism.

Recombination among genomes can occur by a number of different mechanisms. The most familiar one is eukaryotic sex, which occurs by meiosis and fertilization. Many eukaryotic microorganisms, including fungi and protozoa, engage in sexual mixis. Bacteria generally reproduce asexually, but may undergo mixis via conjugation (plasmid-mediated), transduction (virus-mediated), or transformation. Even viruses may recombine when two or more coinfect a single cell.

Unlike mutation, these various mechanisms do not necessarily produce organisms with new genes; instead, they may produce organisms that possess new combinations of genes. This can have very important evolutionary consequences. In the absence of mixis, two or more beneficial mutations can be incorporated into an evolving population only if they occur sequentially in a single lineage. Mixis allows beneficial mutations that occur in separate lineages to be combined and thereby incorporated simultaneously by an evolving population. Thus, mixis may accelerate the rate of adaptive evolution by bringing together favorable combinations of alleles.

Natural selection

One of the most conspicuous features of biological evolution is the evident 'fit' (adaptation) of organisms to the environments in which they live. For centuries, this match between organism and environment was taken as evidence for the design of a Creator. But in 1859, Charles Darwin published 'The Origin of Species', in which he set forth the principle of adaptation by natural selection. This principle follows logically from three simple premises. First, variation among individuals exists for many phenotypic traits. Second, these phenotypic traits influence survival and reproductive success. Third, phenotypic variation in those characters that affect survival and reproductive success is heritable, at least in part. (Many phenotypic traits are subject to both genetic and environmental influences.) Hence, individuals in later generations will tend to be better adapted to their environment than were individuals in earlier generations, provided that there is heritable phenotypic variation and the environment has not changed too much in the intervening time. (Environments do sometimes change, of course, and when this happens a population or species may go extinct if it cannot adapt to these changes.)

Darwin himself did not know about the material basis of heredity (DNA and chromosomes), nor did he even understand the precise causes of heritable variation among individuals (mutation and mixis). What he clearly understood, however, was that this heritable variation did exist and its causes could be logically separated from its consequences for the reproductive success of individuals and the resulting adaptation of species to their environments.

Darwin's theories were influenced, in part, by his observations on the practices of breeders of domesticated animals and plants. These practices are now commonly referred to as artificial selection. It is useful to distinguish between artificial and natural selection, and to relate this distinction to experimental evolution in the laboratory. Under artificial selection, organisms are chosen by a breeder, who allows some but not all individuals to survive and reproduce. Individuals are thus selected on the basis of particular traits that are deemed desirable to the breeder. By contrast, under natural selection, no one consciously chooses which individuals within a population will survive and reproduce and which will not. Instead, the match between organismal traits and environmental factors determines whether or not any given individual will survive and reproduce.

At first glance, one might regard laboratory studies as examples of artificial selection. Such usage, however, would not reflect the critical distinction between artificial and natural selection, that is, whether a breeder or the environment determines which individuals survive and reproduce. In experimental evolution, an investigator typically manipulates environmental factors, such as temperature and resource concentration, but he or she does not directly choose which individuals within an experimental population will survive and reproduce. Instead, natural selection in the laboratory, like natural selection in the wild, depends on the match between organismal traits and environmental factors.

Genetic drift

The frequency of genes within populations, and hence also the distribution of phenotypic traits, may change not only as a consequence of natural selection, but also as a consequence of the random sampling of genes during transmission across generations. This random sampling is called genetic drift. In practice, it can be difficult to distinguish between natural selection and genetic drift, although statistical methods have been developed that may allow one to distinguish between these forces by comparing DNA sequences among related strains. Alternatively, by using microorganisms to study evolution experimentally, it is possible to compare the survival and reproductive success of different genotypes that are placed in direct competition with one another. With proper replication of such experiments, one can distinguish systematic differences in survival and reproductive success from chance deviations due to drift.

Experimental Tests of Fundamental Principles

Two key principles of evolutionary theory are the randomness of mutation and adaptation by natural selection. According to the former, mutations occur irrespective of any beneficial or harmful effects they have on the individual organism. According to the latter, organisms in later generations tend to be better adapted to their environment than were those in earlier generations, provided that the necessary genetic variation exists and the environment itself does not change.

Random Mutation

For many years, it was known that bacteria could adapt to various environmental challenges. For example, the introduction of bacteriophage into a population of susceptible bacteria often caused the bacterial population to become resistant to viral infection. It was unclear, however, whether mutations responsible for bacterial adaptation were caused directly by exposure to the selective agent, or whether this adaptation was the result of random mutation and subsequent natural selection. Two elegant experiments were performed in the 1940s and 1950s, which demonstrated that mutations existed prior to exposure to the selective agent, so that these mutations could not logically have been caused by that exposure.

Fluctuation test

The first of these experiments was published by Salvador Luria and Max Delbrück in 1943, and it relies on subtle mathematical reasoning. Imagine a set of bacterial populations, each of which grows from a single cell to some large number of cells (N); the founding cells are identical in all of the populations. If exposure to the selective agent causes a bacterial cell to mutate with some low probability (p), then the number of mutants in a population is expected to be, on average, pN. Although this probability is the same for each of the replicate populations, the exact number of mutants in each population may vary somewhat due to chance (just as the number of heads and tails in 20 flips of a fair coin will not always equal exactly 10). Mathematical theory shows that the expected variance in the number of mutants among the set of replicate populations is equal to the average number of mutants under this hypothesis.

Now imagine this same set of populations, but assume that mutations occur randomly, that is, independent of exposure to the selective agent. During each cell generation, there is a certain probability that one of the daughter cells is a mutant. A mutant cell's progeny are also mutants, and so on. According to mathematical theory for this hypothesis, the variance in the number of mutants among the replicate populations should be much greater than the average number of mutants. This large variance comes about because mutations will, by chance, occur earlier in some replicate populations than in others, and each of the early mutations will leave many descendant mutants as a consequence of the subsequent population growth.

Luria and Delbrück designed experiments that allowed them to measure both the average and the variance of the number of mutants in a set of populations of the bacterium *Escherichia coli*. The observed variance was much greater than expected if exposure to the selective agent had caused the mutations. Hence, their results supported the hypothesis of random mutation.

Replica plating experiment

Joshua and Esther Lederberg devised a more direct demonstration of the random origin of bacterial mutations, which they published in 1952. In their experiment, thousands of cells are spread on an agar plate that does not contain the selective agent; each cell grows until it makes a small colony, and the many colonies together form a lawn of bacteria (master plate). By making an impression of this plate using a pad of velvet, cells from all of the colonies are then transferred to several other agar plates (replica plates) that contain the selective agent, which prevents the growth of colonies except from those cells with the appropriate mutation. If mutations are caused by exposure to the selective agent, then there should be no tendency for mutant colonies found on the replica plates to be derived from the same subset of colonies on the master plate. However, if mutations occur during the growth of the colony on the master plate (i.e., prior to exposure to the selective agent), then those master colonies that give rise to mutant colonies on one replica plate should also give rise to mutant colonies on the other replica plates. Indeed, Lederberg and Lederberg observed that master colonies giving rise to mutants on one replica plate gave rise to mutants on the other replica plates. Moreover, they could isolate resistant mutants from those master colonies, without the cells having ever been exposed to the selective agent. This experiment thus demonstrates that the mutations had occurred randomly during the growth of the colony on the master plate.

Adaptation by Natural Selection

In addition to demonstrating the random occurrence of mutations, the fluctuation test and the replica plating experiment both demonstrate adaptation by natural selection. Two other types of experiments also demonstrate adaptation by natural selection. One type is very complicated and involves monitoring the dynamics of mutation accumulation at one genetic locus, which is not under selection, in order to study indirectly what is happening at some other loci, which are under selection.

The other type of experiment that demonstrates adaptation by natural selection involves direct estimation of an evolving population's fitness relative to its ancestor, and it is conceptually quite simple. A population is founded by an ancestral clone, which is also stored in a dormant state (usually at very low temperature). The population is then propagated in a particular environment, and one or more samples are obtained after many generations have elapsed. These derived organisms are placed in direct competition with the ancestral clone under the same defined environmental conditions (after both types have acclimated physiologically to these conditions). If the derived organisms increase their number relative to the ancestral clone, in a systematic and reproducible fashion, then the evolving population has evidently become better adapted to the environment as the result of mutation and natural selection.

To distinguish the derived and ancestral types from one another in a competition experiment, it is usually necessary to introduce a genetic marker that can be scored into one of them. This genetic manipulation necessitates an appropriate control experiment to estimate the effect of the genetic marker on fitness.

Genetic and Physiological Bases of Fitness

The fact that one strain may be more fit than another in a particular environment usually says little or nothing about the causes of that difference. It is interesting to know why one strain is more fit than another in terms of their genotypes and their physiological properties. By using both classical and molecular genetic methods, one can construct genotypes of interest and then determine the effects of their differences on physiological performance and fitness.

Effects of Single and Multiple Mutations

A study by Santiago Elena and Richard Lenski examined systematically the fitness effects of a large set of random mutations. Using transposon mutagenesis, they made 225 genotypes of *E. coli* that each carried one, two, or three insertion mutations. Each genotype was then placed in competition with the unmutated progenitor strain in order to measure the fitness of the mutant relative to the progenitor. Single insertion mutations reduced fitness by about 2%, on average, and there was tremendous variability in the mutational effects, which ranged from approximately neutral to effectively lethal under the conditions where the competitions were performed.

The relationship between average fitness and mutation number was approximately log-linear. That is, subsequent mutations were neither more nor less harmful, on average, than was the first mutation. However, many pairs of mutations interacted strongly with one another, that is, their combined effect on fitness was different from what was expected given their separate effects. (The overall relationship between average fitness and mutation number was approximately log-linear because some interactions were synergistic whereas others were antagonistic.) These data imply that a full understanding of the genetic basis of any organism's functional capacity cannot depend entirely on the step-by-step analysis of individual genes and pathways; instead, a more integrative approach is necessary.

Fitness Effects due to Possession of Unused Functions

A number of studies have used well-characterized bacterial genotypes to examine the effects on fitness caused by the carriage and expression of superfluous gene functions. These studies have measured the relative fitnesses of (1) bacteria with constitutive (high level) and repressed (low level) expression of enzymes for catabolism of carbon sources in media where those resources are not available; (2) prototrophic bacteria (which produce an amino acid or other required nutrient) and auxotrophic mutants (which cannot produce that nutrient) in media where the required nutrients are supplied; (3) phage-sensitive bacteria and resistant mutants when the phage are absent; and (4) antibiotic-sensitive bacteria and resistant genotypes in media that contain no antibiotics.

These studies have often, but not always, demonstrated substantial fitness disadvantages due to possession of unnecessary gene functions. In some cases where such disadvantages have been detected, they are much greater than can be explained by the energetic costs of synthesizing the extra proteins and other metabolites. For example, a study by Daniel Dykhuizen found that the fitness disadvantage associated with synthesis of the amino acid tryptophan, when it was supplied in the medium, was much greater than could be explained on the basis of energetic costs alone. Evidently, the expression of superfluous functions can sometimes have strong indirect effects, which presumably arise through the disruption of other physiological processes.

The idea that microorganisms may have reduced fitness owing to possession of unnecessary functions has two important practical implications. First, in many bioengineering applications, microorganisms are constructed that constitutively express high levels of some compound (e.g., a pharmaceutical) that can be harvested for its commercial value. However, the microorganisms themselves do not benefit from producing that compound, and so any mutant that no longer produces the compound may have a selective advantage. Such a mutation would thus spread through the population and thereby reduce the efficiency of the production process. Second, the spread of antibiotic-resistant bacteria has become a serious concern in public health. It has been proposed that the prudent use of antibiotics, including even the elimination of their use in certain environments (e.g., animal feeds), might favor antibiotic-sensitive bacteria, thereby restoring the efficacy of antibiotics. This proposal rests, in part, on the presumption that resistant bacteria are less fit than their sensitive counterparts, in the absence of antibiotic, due to their possession of the superfluous resistance function. This tradeoff often appears to be the case, but sometimes antibiotic-resistant bacteria evolve solutions that reduce or even eliminate the cost of resistance, thus complicating efforts to contain their spread.

Effects due to Variation in Essential Metabolic Activities

It is clear that the expression of unnecessary metabolic functions is often disadvantageous to a microorganism. An equally important issue concerns the relationship between fitness and the level of expression of functions that are required for growth in a particular environment. This latter issue is more difficult to address experimentally, because it demands careful analyses of subtle differences between strains in their biochemical activities.

Daniel Dykhuizen, Antony Dean, and Daniel Hartl performed a pioneering study to examine the relationships between genotype, biochemical activities in a required metabolic pathway, and fitness. Their study examined growth on lactose by genotypes of *E. coli* that varied in levels of expression of the permease used for active uptake of lactose and the β-galactosidase required for hydrolysis of the lactose. (The genotypes were otherwise essentially identical.) Given that both enzymes are necessary for growth on lactose, how do the activities at each step affect the net flux through this metabolic pathway? And how does net flux affect fitness?

Metabolic control theory consists of mathematical models that describe the dynamics across multiple steps in a biochemical pathway. Using this theory, Dykhuizen and colleagues predicted how the net flux through this pathway would depend on the activities of the permease and β-galactosidase enzymes, and they measured these activities for several different genotypes using biochemical methods. They then predicted that the relative fitness of any two genotypes would be directly proportional to their relative fluxes if lactose was provided as the sole energy source. In order to test the theory and its predictions, they estimated the relative fitnesses of the various genotypes in a medium in which lactose was the sole source of energy for growth. The observed fitnesses were very close to the predicted values.

Dykhuizen and Dean have also successfully extended this mechanistic approach to predicting fitness in competition for mixtures of lactose and glucose. However, the results to date (for both single and mixed sugars) were obtained with genotypes in which gene regulation was eliminated in order to simplify the analysis. An important challenge for the future is to include the complex dynamical effects of gene regulation in the models and experiments.

Effects of Genetic Background

It is obvious that the fitness effects caused by particular genetic differences strongly depend on the environment. For example, an antibiotic resistance gene function that is essential for survival and replication of a bacterium in the presence of antibiotic may hinder growth in an antibiotic-free environment. Similarly, the fitness effect that is due to a particular gene function may often depend on the genetic background in which that gene is found.

For example, one study found that different alleles at the 6-phosphogluconate dehydrogenase (6-PGD) locus in *E. coli* had similar fitnesses in a gluconate-limited medium, provided that these alleles were present in a genetic background that also encoded an alternative metabolic pathway for 6-phosphogluconate utilization. In a genetic background where this alternative pathway was defective, however, these alleles had quite variable fitnesses in that same medium.

In another study with *E. coli*, it was observed that the selective disadvantage associated with bacteriophage resistance mutations in a virus-free environment was substantially reduced during several hundred generations of experimental evolution. This fitness improvement resulted from secondary mutations in the genetic background that compensated for the maladaptive side effects of the resistance mutations, but which did not diminish the expression of resistance.

Other studies, including one by Stephanie Schrag, Veronique Perrot, and Bruce Levin, have demonstrated similar compensatory evolution among antibiotic-resistant bacteria. When bacteria resistant to an antibiotic first arise, they are typically less fit in the absence of the antibiotic, thus helping to control their proliferation. Over time, however, the evolving bacteria become very good competitors in the absence of antibiotic while retaining their resistance to antibiotic. That is, the bacteria find ways to 'have their cake and eat it, too'. Such compensatory evolution unfortunately makes it more difficult to devise strategies to manage the spread of antibiotic-resistant pathogens.

Speciation and Genetic Exchange

As populations diverge from one another over time, the potential for gene flow between them lessens. The increased barriers to gene flow between more distantly related organisms reflect both molecular processes and selective factors. At the molecular level, highly diverged DNA sequences recombine less efficiently than similar sequences. From the standpoint of selection, genes that evolved in one lineage may not function as well when they are moved into a different lineage, where they must function within a different physiological context. Therefore, when two lineages diverge and adapt to different environments for a sufficiently long time, they become separate species. A recent experiment by Jeremy Dettman and colleagues examined the effects of adaptation to different environments on the fitness of progeny in the yeast *Saccharomyces cerevisiae*. The researchers generated hybrid progeny by sexually crossing yeast strains, using three different types of strain pairings: (1) two strains that had independently evolved in the same environment; (2) two strains that evolved in different environments; and (3) one evolved strain and its ancestor. The hybrid progeny of strains that had both evolved in the same environment were more fit than the other types of hybrids, while the least fit progeny resulted from crosses between strains that evolved in different environments. These results demonstrate that incipient speciation can occur rapidly as populations adapt to different environments and diverge from one another, so that their hybrid progeny are not well adapted to the environment of either parent.

Over time, another process, called reinforcement, may occur that further separates and isolates two incipient species. Reinforcement occurs when organisms evolve mating preferences so that they become less likely to mate with individuals that are ecologically and genetically distinct, thereby avoiding the production of mal-adapted hybrid offspring. In another recent experiment with yeast, Jun-Yi Leu and Andrew Murray showed the rapid evolution of mate discrimination. After only a few dozen rounds of selection for assortative mating, yeast cells had evolved that were five times more likely to mate with their own type than with the ancestor.

Genomic Analyses of Experimental Evolution

As technologies change, so do the questions that biologists can address. Our understanding of the extent and patterns of genetic diversity has grown with each new advance. For many years, geneticists had to rely on differences that were visible to the naked eye – for example, the round and wrinkled seeds, and white and purple flowers, of the pea plant that Gregor Mendel studied to discover the basic laws of inheritance in plants and animals. Several decades ago, biochemists discovered they could discern subtle differences in proteins, caused by mutations in the genes that encode them, by examining how the variant proteins moved through gels that were subject to electrical fields. This approach revolutionized biology by demonstrating tremendous levels of genetic diversity in almost every species that was investigated, from bacteria to humans. More recently, the ability to sequence DNA molecules now allows scientists to examine and compare the hereditary information of organisms at its most fundamental level. It has become possible to sequence genes isolated directly from the environment, allowing ecologists to examine patterns of diversity even among microbes that have never been directly observed or cultured. It has also become possible to sequence the entire genome of any organism. With improving technologies and declining costs, it is becoming feasible to pursue whole-genome sequencing to discover and investigate all of the genetic changes that occur during laboratory-based evolution experiments.

A fascinating question in evolution – and one that has benefited from the improving genomic technologies – concerns the reproducibility of evolutionary outcomes. The late paleontologist Stephen Jay Gould imagined the thought experiment of ‘replaying life’s tape’ to address this question. Of course, it is impossible to rerun evolution for billions of years on the scale of an entire planet, but with microbes one can perform careful experiments to ask whether replicated populations that start with the same ancestor and evolve in the same environment will arrive at the same or different solutions to the challenges they face. A long-term study by Richard Lenski and colleagues has monitored the evolution of 12 initially identical populations of *E. coli* as they evolved in and adapted to the same laboratory environment for tens of thousands of generations. They identified many parallel, or repeatable, changes in the phenotypes of the evolving lineages, including improved competitiveness in the glucose-based medium where they evolved, reduced performance on certain other sugars including ribose and maltose, larger cell volumes, altered supercoiling of their chromosomes, and so on. Robert Woods, Richard Lenski and colleagues further analyzed this experiment by sequencing many genes in the ancestor and all 12 evolved lines. They found several genes that had acquired mutations in most or all of the evolved lines, even though most genes showed no substitutions at all in any of the lines. These data indicate, therefore, a high degree of evolutionary reproducibility even at the genetic level.

Conceptually related work has now been extended to the entire genome in experiments with evolving viruses and bacteria. Holly Wichman, James Bull and colleagues have documented striking evolutionary reproducibility in the bacteriophage φX174 that, in this case, often extends to the nucleotide level. Christopher Herring, Bernhard Palsson and colleagues have used new methods for rapidly resequencing entire bacterial genomes to identify several genes that changed repeatedly in short-term experiments in which *E. coli* evolved in and adapted to a glycerol-based medium.

Genome-level resequencing was also used by Gregory Velicer and colleagues to discover mutations that arose during a two-stage evolution experiment with the bacterium *Myxococcus xanthus*. This species is fascinating because cells, when they are starving, form multicellular aggregations. Some of the cells in these aggregations differentiate into stress-tolerant spores that can be dispersed and germinated in more favorable environments, but the majority of cells die while forming a mound-like structure that elevates the spores and helps them disperse. In the first stage of their experiment, Velicer and colleagues evolved *M. xanthus* strains that ‘cheated’ on their progenitors during aggregation and differentiation. By themselves, these cheaters were very poor at aggregating and producing spores, but when they were mixed with their cooperative progenitors, the cheaters were disproportionately likely to end up among the surviving spores. In the second stage of this evolution experiment, Velicer and colleagues found one cheater-derived strain that had recovered the ability to cooperate, although its mechanism of cooperation was somewhat different from the ancestral cooperator. By resequencing the genomes of both the evolved cheater and the restored cooperator, and comparing them to the ancestral genome, they found 14 mutations that led to the cheater, while only a single mutation – although obviously a very important one – was responsible for the evolved restoration of the cooperative behavior.

Genetic Variation within Populations

In nature, there exists abundant genetic variation in most species, including microorganisms. Some of this variation exists within local populations, while other variation may distinguish one population from another. This section describes some of the dynamic processes that influence genetic variation within populations.

Transient Polymorphisms

A population is polymorphic whenever two or more genotypes are present above some defined frequency (e.g., 1%). A polymorphism arises whenever an advantageous mutation is increasing in frequency relative to the ancestral allele. This type of polymorphism is called transient, because eventually the favored allele will exclude the ancestral allele by natural selection.

Selective Neutrality

At the other extreme, some polymorphisms may exist almost indefinitely precisely because the alleles that are involved have little or no differential effect on fitness. Such selectively neutral alleles are subject only to genetic drift. Daniel Dykhuizen and Daniel Hartl sought to determine whether some polymorphic loci in natural populations of *E. coli* might exist because of selective neutrality, or whether other explanations are needed. To that end, naturally occurring alleles at certain loci were moved into a common genetic background, and the fitness effects associated with the various alleles were determined. Even when the bacteria were grown under conditions where growth was directly dependent on the particular enzymes encoded by these loci, there were often no discernible effects on fitness due to the different alleles. These results therefore support the hypothesis that random genetic drift is responsible for some of the genetic variation that is present in natural populations.

Frequency-Dependent Selection

In the course of growth and competition in a particular environment, microorganisms modify their environment through the depletion of resources, the secretion of metabolites, and so on. When this happens, the relative fitness of genotypes may depend on the frequency with which they are represented in a population, and selection is said to be frequency-dependent. Frequency-dependent selection can give rise to several different patterns of genetic variation.

Stable equilibria

Two (or more) genotypes can coexist indefinitely when each has some competitive advantage that disappears as that genotype becomes more common. In that case, each genotype can invade a population consisting largely of the other genotype but cannot exclude that other genotype, so that a stable equilibrium results.

Several different ecological interactions can promote a stable equilibrium. For example, an environment may contain two different resources. If one genotype is better at exploiting one resource and another genotype is superior in competition for the second resource, then whichever genotype is rarer will tend to have more resource available to it, thereby promoting their stable coexistence. In some cases, a resource that is essential for one genotype may be produced as a metabolic by-product of growth by another genotype; such interactions are often called cross-feeding. Stable coexistence of genotypes in one population can also occur when the environment contains a population of predators (or parasites); predator-mediated coexistence requires that one of the prey genotypes be better at exploiting the limiting resource while the other prey genotype is more resistant to the predator. The evolution of two or more stably coexisting bacterial types from a single ancestral type has been demonstrated in several experiments involving both cross-feeding and predator–prey interactions.

A striking example of the rapid evolution of several stably coexisting genotypes comes from an experiment on *Pseudomonas fluorescens* performed by Paul Rainey and Michael Travisano. The experiment started with a single clone that was placed in a static (unshaken) flask containing a nutritionally rich liquid medium. Within a few days, the bacteria evolved into three distinct genotypes that could be distinguished by the appearance of their colonies, and these types then coexisted with one another. By reconstituting the various combinations of these three types, the authors showed that each type had a selective advantage when it was rare relative to one or both of the other types. As a consequence, each genotype could invade and coexist with the others, so that a stable community was formed. However, if the medium and cells were thoroughly mixed by physically shaking the flask, then this coexistence was disrupted and one genotype prevailed. The three genotypes coexisted in the static flask because they had evolved different abilities to exploit gradients, such as in oxygen concentration, which were generated by the organisms' metabolic activities in concert with the physical environment. When these gradients were eliminated by continually shaking the flask, stable coexistence of the three genotypes was impossible.

Unstable equilibria

Those ecological interactions that promote the stable coexistence of two or more genotypes contribute to the maintenance of genetic variation in populations. However, certain ecological interactions give rise to unstable equilibria. An unstable equilibrium exists when each of two genotypes prevents the other from increasing in number. Such interactions do not promote polymorphisms within a local population. However, they may contribute to the maintenance of genetic differences between populations, because neither type can invade a resident population of the other type.

One form of ecological interaction that can give rise to an unstable equilibrium is interference competition. This interaction occurs when one genotype produces a toxic substance that inhibits the growth of competing genotypes; it is distinguished from exploitative (scramble) competition, which occurs by the depletion of resources. Many microorganisms secrete such toxins, including fungi that produce antibiotics. Similarly, some strains of *E. coli* produce colicins that kill some competing strains of *E. coli*, but to which the producing genotypes are immune. The producing types, when common, make so much toxin that they can eliminate a sensitive strain that is more efficient in exploitative competition. When the producing cells are rare, however, the cost of their colicin production is greater than the benefit of the resource that becomes available by the killing of sensitive cells, and the producing type loses out to the more efficient colicin-sensitive competitor. The outcome of competition between colicin-producing and sensitive strains also depends on the physical structure of the environment, as shown in experiments by Lin Chao and Bruce Levin. In particular, the advantage shifts to the colicin-producing cells on agar surfaces, even when they are rare, because the resources made available by the killing action of colicins accrue locally to the producers, rather than being dispersed evenly as in a well-stirred liquid medium.

Nontransitive interactions

In some cases, the frequency-dependent interactions among three or more genotypes are so complex that they become nontransitive. For example, genotype *A* out-competes genotype *B*, and genotype *B* out-competes genotype *C*, but genotype *C* out-competes genotype *A*. A familiar example is the game of rock-paper-scissors, in which rock beats scissors, scissors beat paper, and paper beats rock. Benjamin Kerr, Brendan Bohannan and colleagues studied an example of this game played by three strains of *E. coli* on the surface of agar plates. One strain produced a toxic colicin, while the second strain was sensitive to the colicin. The third strain was resistant to the colicin, although it did not produce any toxin. In pairwise interactions, the colicin-producing strain out-competes the sensitive strain by poisoning it. The resistant strain out-competes the producing strain by not wasting resources to produce the colicin, as both strains are resistant to it. The sensitive strain, in turn, out-competes the resistant strain because there is no colicin around, and the resistant strain pays a fitness cost for its resistance. This research team further showed, both by experiments and in computer simulations, that the three strains could coexist with one another only in a spatially structured environment, such as on the surface of an agar plate. By contrast, in a well-mixed liquid medium, one strain dominates, although the identity of the winner depends on the initial abundances.

As another example, Charlotte Paquin and Julian Adams found nontransitive interactions in populations of the yeast, *S. cerevisiae*, that were evolving in chemostats fed with glucose as a sole carbon source. Nontransitive interactions can lead to situations in which the average fitness of an evolving population declines relative to some distant ancestor, even though each successive dominant genotype has increased fitness relative to its immediate predecessor. Indeed, Paquin and Adams observed precisely this phenomenon.

Evolution in a Changing Environment

Most evolution experiments operate within a controlled, defined environment, which facilitates the analysis and interpretation of these experiments. However, many environments change over time owing to either external processes, such as a change in temperature, or internal processes, such as resource depletion or coevolution of interacting species. This section examines research on the evolution of bacteria in response to prolonged resource deprivation, while the next section will address the coevolution of interacting species.

When bacteria are inoculated into fresh medium, they grow exponentially until they exhaust the available nutrients, at which point the cells typically enter a ‘stationary’ phase during which they neither grow nor die, at least for many hours or even days. However, if the bacteria are left in the nutrient-depleted medium indefinitely, they eventually enter a death phase, although not all the cells necessarily die even after a very long time. Roberto Kolter and Steven Finkel studied the ecological and evolutionary dynamics in long-term cultures of *E. coli* that were grown in a nutrient-rich medium and then left for over five years without additional nutrients, except that sterile water was added occasionally to compensate for evaporation. Some 99% of the cells died over the first few weeks of the death phase. If that rate of decline had continued throughout the experiment, then there would soon have been no survivors at all. However, Kolter and Finkel found that the surviving cells entered a sort of second stationary phase, in which the population declined only very slowly. During this period, the rates of cell division and death were nearly equal, so that the population was dynamic rather than static. Moreover, the environment itself changed continually owing to the buildup and breakdown of metabolic by-products released by both living and dying cells. This changing environment, in turn, favored mutants that were better able to survive and grow under these challenging conditions than was the strain used to begin this experiment. The authors called these winners 'GASP' mutants because they had a growth advantage in stationary phase (GASP). Further analyses of this system found multiple waves of GASP mutants that arose and swept to high frequency in the population, only to be displaced later by other, even tougher, mutants. Thus, many different mutations can produce GASP phenotypes, and the various mutants differ in the details of their physiological and ecological advantages.

Coevolution of Interacting Genomes and Species

Microorganisms in nature rarely, if ever, exist as single species, as they are usually studied in the laboratory. Rather, they exist in complex communities of many interacting populations. Some interactions are exploitative, such that one population makes its living by parasitizing or preying upon another population. Other interactions are mutualistic, such that each population obtains some benefit from its association with the other. In many cases, these interactions are plastic both genetically and ecologically. For example, a single mutation in a bacterium may render it resistant to lethal infection by a bacteriophage. And a plasmid that confers antibiotic resistance may be beneficial to its bacterial host in an antibiotic-containing environment but detrimental in an antibiotic-free environment.

As a consequence of this variability, microorganisms have proven useful for investigating questions about the coevolution of interacting populations. Are there evolutionary 'arms races' between host defenses and parasite counterdefenses? Why are some parasites so virulent to their hosts, whereas others are relatively benign? How can mutualistic interactions evolve, if natural selection favors 'selfish' genes that replicate themselves even at the expense of others?.

Exploitative Interactions

A number of studies have demonstrated the stable coexistence of virulent bacteriophage (lytic viruses) and bacteria in continuous culture. In these studies, the virus population may hold the bacterial population in check at a density that is several orders of magnitude below the density that would be permitted by the available resource if viruses were not present. In most cases, however, bacterial mutants eventually appear that are resistant to the virus, and these mutants have a pronounced selective advantage over their virus-sensitive progenitors. The proliferation of bacteria that are resistant to infection by the original virus provides a selective advantage to host-range viral mutants, which are capable of infecting the resistant bacteria. Thus, one can imagine, in principle, an endless 'arms race' between resistant bacteria and extended host-range viruses.

In fact, there are constraints that often preclude this outcome. Experiments performed by Lin Chao, Richard Lenski, and Bruce Levin using *E. coli* and several lytic viruses found that bacterial mutants eventually evolved for which it was very difficult or impossible to isolate corresponding host-range viral mutants. This asymmetry may arise because bacterial resistance can occur via mutations that cause either the structural alteration or the complete loss of certain receptors on the bacterial surface, whereas viral host-range mutations can counter only the former. Despite this asymmetry, these experiments also demonstrated that the virus population often persisted because the virus-resistant bacterial mutants were less efficient than their sensitive progenitors in competing for limiting resources. In such cases, the result was a dynamic equilibrium, in which the growth-rate advantage of the sensitive bacteria relative to the resistant mutants was offset by death due to viral infection. Such tradeoffs between competitiveness and resistance commonly occur, because the same receptors used by viruses to adsorb to the cell envelope often serve to transport nutrients into the cell or to maintain its structural integrity.

A widely held belief is that a predator or parasite that is too efficient or virulent will drive its prey or host population extinct, thereby causing its own demise. However, virulent phage often coexist with bacteria, even though successful reproduction of the virus is lethal to the infected bacterium. Moreover, the process of natural selection does not involve foresight, so the mere prospect of extinction cannot deter the evolution of more efficient predators or more virulent parasites. Nevertheless, there exist many viruses (lysogenic and filamentous bacteriophage) that are replicated alongside the host genome, and whose infections, although deleterious, are not necessarily lethal. These viruses, as well as conjugative plasmids, have life cycles that include both horizontal (infectious) and vertical (intergenerational) transmission.

At present, the evolutionary forces that favor these alternative modes of transmission are not fully understood. One factor that is thought to be important is the density of hosts. If susceptible hosts are abundant, then there are many opportunities for horizontal transmission. In that case, selection favors those parasites that replicate and infectiously transmit themselves most rapidly, regardless of the consequences of these activities for the host's fitness. On the other hand, if susceptible hosts are scarce, then horizontal transmission becomes infrequent. Vertical transmission, by contrast, does not depend upon the parasite or its progeny finding another host. Instead, the success of a vertically transmitted parasite is determined by the success of its infected host. The greater the burden that such a parasite imposes on its host, the slower the host can reproduce its own genome and that of the parasite. Hence, when the density of susceptible hosts is low, selection may favor those parasites that minimize their replicative and infectious activities, and thereby minimize their deleterious effects on the host.

Two studies sought to test this hypothesis by manipulating the supply of susceptible hosts. One experiment, by Jim Bull and colleagues, with a filamentous bacteriophage supported the hypothesis. The other study, by Paul Turner and Richard Lenski, used a conjugative plasmid, and their experiment did not support that hypothesis, for reasons that are unclear. However, both studies demonstrated genetically encoded tradeoffs between the parasites' rates of horizontal and vertical transmission. That is, parasites that were transmitted between individual hosts at higher rates reduced the host's growth rate – and hence their own vertical transmission – more severely than did parasites that were infectiously transmitted at lower rates.

In addition to subtle changes in the interaction between a virus and its current host, evolution can occur when a virus evolves the ability to infect a different host species. Most viruses are severely constrained in the organisms that they can infect because they must enter their host via specific proteins or other receptors on the host's cell surface, and these receptors typically differ between host species. Two viral genomes can only recombine genetically when they both infect the same host. Therefore, if a virus evolves the new ability to infect a different host species and, moreover, loses its ability to infect its previous host, then this host shift may lead to speciation, whereby a single virus population eventually evolves to become two distinct types of virus.

A study by Wayne Crill, Holly Wichman, and Jim Bull examined the adaptation of a virus to two different host species. They alternated a bacteriophage population between two bacterial hosts, *E. coli* and *Salmonella enterica*. The experiment was designed so that the virus saw only 'naïve' bacteria that had not previously been exposed to the virus; hence, the bacteria could not evolve resistance that might complicate their analysis of the evolving virus. Because the viral genome is small, the authors could completely sequence several isolates of the virus after each round of their adaptation to the alternating hosts. The authors found a single base pair in the virus genome that alternated in perfect concordance with the host species to which the virus had most recently adapted. Owing to this and other mutations, viral growth rate was always higher on the current host than on the alternate host. However, there was a strong asymmetry in this host-specific adaptation. Specifically, adaptation to *S. enterica* led to reduced viral growth on *E. coli*, but adaptation to *E. coli* did not cause a comparable reduction in growth on *S. enterica.* This finding has potential relevance for the production of vaccines, because it is common practice to produce weakened versions of infectious viruses by adapting them to new cell types, and then using these attenuated viruses in vaccines. If viral adaptation to a new host type does not always impair viral growth on its usual host, then it becomes all the more necessary to test carefully the safety of such putatively attenuated viruses. More generally, these results show that even single mutations can sometimes allow viruses to infect new host species, a finding that is of interest not only for understanding evolution but also to public health.

Mutualistic Interactions

It has been proposed that many mutualisms evolved from formerly antagonistic interactions. In fact, mathematical models predict that, at low host densities, genetic elements such as plasmids and phage can persist only if they are beneficial to their host. Many plasmids encode functions useful to their bacterial hosts, including resistance to antibiotics, catabolic pathways, production of bacteriocins, and so on. Also, some plasmids are unable to promote conjugation, thus relying exclusively on vertical transmission. This limitation requires that the plasmid does not harm its host, because any plasmid-free derivative would otherwise out-compete those cells that carry a costly plasmid. Indeed, several studies have found unexpected competitive advantages for bacteria that are infected by plasmids, transposons, and even temperate phage, relative to cells that are not infected but otherwise genetically identical.

Two studies have even demonstrated the evolution of mutualistic interactions from formerly antagonistic associations. Kwang Jeon showed that the growth of the protist *Amoeba proteus* was initially greatly reduced by a virulent bacterial infection. The harmful effects of the bacteria were diminished, however, by propagating the infected amoebae for several years. In fact, the amoebae eventually became dependent on the bacterial infection for their viability. In another study, Judith Bouma and Richard Lenski found that a certain plasmid initially reduced the fitness of its *E. coli* host in antibiotic-free medium. However, the plasmid enhanced the fitness of its host in this same medium after 500 generations of experimental evolution. Interestingly, the mutation responsible for the newly evolved mutualistic interaction was in the host, not in the plasmid. Both of these experiments show that hosts can become dependent on, or otherwise benefit from, formerly parasitic genomes, thus giving rise to mutualistic interactions.

Evolution of New Metabolic Functions

Microbes exhibit a tremendous diversity of metabolic activities, some of which function in degradative pathways (catabolism) while others work in synthetic pathways (anabolism). How has this diversity evolved? One area of research in the field of experimental evolution seeks to elucidate the various processes by which microorganisms can acquire new metabolic functions. This research is timely as humans seek to employ certain microbes that degrade toxic pollutants in the environment, and to harness others that may be useful in the production of biofuels.

Acquisition by Gene Transfer

Perhaps the simplest way in which a microorganism can acquire some new metabolic function is by gene transfer from another microorganism that already encodes that function. For example, antibiotic resistance functions are often encoded by plasmids, which are transmitted from donors to recipients by conjugation. However, acquiring new functions by gene transfer is not always so simple. Biodegradation of certain recalcitrant compounds may require the complex coordination of several steps in a biochemical pathway, which are encoded by complementary genes from two (or more) different microorganisms. The acquisition of activities that depend on such pathways may require not only genetic exchange, but also subsequent refinement of the new function by mutation and natural selection.

Changes in regulatory and structural genes

In a number of studies, microorganisms have been shown to evolve new metabolic functions without any horizontal gene exchange. The evolution of these new functions often occurs by selection for mutations in existing regulatory or structural genes that previously encoded some other function. For example, the bacterium *Klebsiella aerogenes* cannot normally grow on the sugar D-arabinose, although it does possess an enzyme, isomerase, that is able to catalyze the conversion of D-arabinose into an intermediate, D-ribulose, which can be further degraded to provide energy to the cell. This isomerase is normally expressed at a very low level that does not permit growth on D-arabinose. Robert Mortlock and colleagues demonstrated that mutations in regulatory genes that increase the level of expression of this isomerase are sufficient to enable *K. aerogenes* to grow on D-arabinose. They also showed that the ability to grow on D-arabinose could be further improved by certain mutations in the structural gene that change the amino acid sequence of the isomerase in ways that improve its efficiency in converting D-arabinose into D-ribulose.

This study, as well as other experiments and comparative analyses, show that the evolution of new metabolic functions often involves ‘borrowing’ gene products that were previously used for other functions. It is not surprising that this process may sometimes also encroach upon, and disrupt, the previous function. Such encroachment could, in turn, favor gene duplication, a type of mutation whereby a single copy of an ancestral gene gives rise to two copies, each of which may then evolve toward different metabolic capabilities.

Evolution of Genetic Systems

The process of adaptation by natural selection requires genetic variation in traits that influence the survival and reproduction of organisms. As discussed earlier, the two sources of genetic variation are mutation and mixis. Rates of mutation and mixis depend not only on environmental factors (e.g., ultraviolet irradiation), but also on properties of the ‘genetic system’ intrinsic to the organism itself. Here, genetic system refers to all those aspects of the physiology, biochemistry, and reproductive biology of an organism that affect rates of mutation and mixis. For example, organisms have mechanisms of varying efficacy to promote the accurate replication and repair of their DNA. And while sex is an integral part of reproduction for some organisms, many others reproduce asexually, so that the progeny are usually identical to their parent and siblings.

Some of the most interesting questions in evolutionary biology concern the significance and evolutionary consequences of alternative genetic systems. Why do some organisms reproduce sexually, whereas others reproduce asexually? If mutation generates variation that is necessary for adaptation by a population, but most mutations have deleterious effects on the individual, then what mutation rate is optimal? Might organisms somehow be able to choose only those mutations that are beneficial to them, given their present circumstances?

Sexuality and Mixis

Sexual reproduction imposes several costs relative to asexual reproduction. These costs include finding a mate, the risk of disease transmission, and, in higher organisms, the genetic dilution that occurs because a female produces an offspring that carries only half of her genes. Therefore, biologists have long sought to understand the advantages for sexual reproduction that could overcome these disadvantages. Numerous hypotheses have been proposed, and all of them depend, in one way or another, on the genetic variation that results from mixis. Most efforts to test these hypotheses have relied on comparing distributions of sexual and asexual organisms to find variables that correlate with reproductive mode. However, several experiments have tested the evolutionary consequences of mixis using microbes in which one can manipulate the extent of intergenomic recombination.

For example, mixis in viruses can be experimentally manipulated by varying the multiplicity of infection (MOI) of host cells, since recombination of viral genotypes can occur only if two or more viruses infect the same host cell. An experimental study by Russell Malmberg compared the rate of adaptive evolution in bacteriophage populations propagated at high and low MOI; the total number of viruses per population was standardized for both treatments. The average fitness increased more rapidly under the high-MOI (equal to high recombination) treatment than under the low-MOI (equal to low recombination) treatment. This result is consistent with the hypothesis that sexual populations can adapt more rapidly than asexual ones because two or more beneficial mutations can be incorporated simultaneously in the former, but only sequentially in the latter.

Another experiment indicates that the benefit of mixis in accelerating adaptive evolution may depend on the environment. Matthew Goddard, Charles Godfray, and Austin Burt compared the rate of fitness improvement in evolving sexual and asexual populations of the yeast *S. cerevisiae*. By deleting two genes in the yeast, they were able to construct an asexual version that could not undergo meiosis. They then established separate populations of sexual and asexual yeast in both harsh and benign environments, and allowed the yeast to evolve for several hundred generations. Neither sexual nor asexual strains showed significant improvement in the benign environment, implying that they were already well adapted to those conditions. However, both strains adapted to the harsh environment, with the rate of improvement consistently greater for the sexual than the asexual populations. This experiment suggests, therefore, that genetic recombination can be especially important for adaptation to stressful and changing environments.

Some experiments have suggested that another advantage of mixis may occur when the rate of deleterious mutation is high and the effective population size is very small. Such conditions may apply especially to microorganisms with high error rates during replication (e.g., RNA viruses) or those with large genomes (e.g., protozoa), if their populations experience periodic 'bottlenecks'. In these cases, deleterious mutations tend to accumulate in asexual lineages, a process called 'Muller's ratchet' (after the geneticist, H. J. Muller, who first described this phenomenon). However, even occasional mixis can purge lineages of their accumulated load of deleterious mutations, as demonstrated by Lin Chao and colleagues using a segmented RNA virus that infects bacteria. This effect occurs because two recombining genomes may each complement the deleterious mutations that are present in the other, thereby generating some progeny that have a reduced load of deleterious mutations (as well as other progeny with an increased load, which will be removed by natural selection).

In some cases, mixis might not be an adaptation to recombine genes but rather a coincidental consequence of the movement between cells of parasitic entities. In many bacteria, for example, mixis occurs only when cells are infected by viruses (transduction) or plasmids (conjugation). The new combinations of chromosomal genes that sometimes result from such infections may occasionally be advantageous. However, one cannot view phage and plasmids as benevolent agents of bacterial carnal pleasure, because these infectious agents often harm or even kill their hosts.

Evolutionary Effects of Mutator Genes

' Mutator' genes increase the mutation rate throughout an organism's genome by disrupting DNA repair functions. Experiments performed by Lin Chao and others have investigated the effect of mutator genes on bacterial evolution. These studies have revealed a pattern that seems, at first glance, rather curious. When a mutator gene is introduced in a population above a certain initial frequency (e.g., 0.1%), it tends to increase in frequency over the long term. However, if that same gene is introduced at a frequency below that threshold, then it typically goes extinct.

What causes this curious effect? In a sense, there is an evolutionary race between two clones, with and without the mutator gene, to see which one gets the next beneficial mutation. The rate of appearance of new mutations for each clone depends on the product of its population size, N, and its mutation rate, u. When the ratio of mutation rates for the mutator and nonmutator clones, u'/u, is greater than the inverse ratio of their population sizes, N/N', then the mutator clone is more likely to have the next beneficial mutation and thus prevail over the long term. When u'/u, is less than N/N', the nonmutator clone, by virtue of its greater numbers, is likely to produce the next beneficial mutation and thereby exclude the mutator clone.

However, this explanation presents a problem for understanding the evolution of mutators in nature, where they are moderately prevalent in some circumstances. If mutator genes are useful only when they are common, then how do they become common in the first place? Mathematical models and evolution experiments with bacteria indicate that a process called 'hitchhiking' can resolve this paradox. In hitchhiking, a deleterious mutation (such as the one that disrupts DNA repair) gets carried along to high frequency if it is genetically linked to a beneficial mutation. In bacteria, which usually have a single chromosome and reproduce asexually, the entire genome is effectively linked. Moreover, a mutator gene is more likely than any other deleterious mutation to be associated with a new beneficial mutation because of the high mutation rate it causes. It is unlikely that any particular mutation that produces a mutator will yield a beneficial mutation that allows the mutator to hitchhike. Given enough time, however, one 'lucky' mutator may do so, and the mutator gene can then increase in frequency by hitchhiking with the beneficial mutation that it caused.

It has also been proposed that mutator genes may be more common in pathogenic bacteria than in their nonpathogenic counterparts. The idea is that pathogenic bacteria face especially rapidly changing selective conditions owing to immunological and other host defenses. By having a high mutation rate, pathogens would have a better chance of evolving a counterdefense. This explanation also fits well with the hitchhiking hypothesis, because every change in the host that favors a new mutation in the pathogen population creates an opportunity for a mutator allele to hitchhike to high frequency. By contrast, for an organism living in a constant environment to which it is already adapted, beneficial mutations would be much rarer and hence there would be fewer opportunities for a mutator gene to hitchhike. Antonio Oliver, Fernando Baquero, Jesús Blázquez, and colleagues examined the frequency of mutator genes in samples of *Pseudomonas aeruginosa*, a bacterial species that often causes chronic (long-term) lung infections in people with cystic fibrosis. This same species is common in the environment, and it can cause acute (short-term) infections in patients with severe burns or otherwise weakened immune systems. The authors documented a much higher frequency of mutator clones in samples from patients with chronic infections, which supports the hypothesis that frequent changes in host immunity and antibiotic regimens can promote the evolution of high mutation rates.

An experimental study by Csaba Pal, Angus Buckling, and colleagues examined the effect of viral parasites on the evolution of mutation rates in bacteria. They found that *P. fluorescens* that were coevolving in environments with bacteriophage present were much more likely to become mutators than were bacteria that were evolving in the absence of the bacteriophage. This study also supports the general hypothesis that rapidly changing environments – including coevolving parasites as well as hosts – can favor the evolution of elevated mutation rates.

Thus, aspects of genetic systems that increase variation – whether by mutation or mixis – may accelerate adaptive evolution. On the other hand, mutation and mixis can also break down genotypes that are already well adapted to particular environments. The evolution of genetic systems may often reflect the balance between these opposing pressures.

Directed Mutations?

The experiments of Luria and Delbrück and the Lederbergs demonstrated that mutations arose before bacteria were exposed to a selective agent, and thus the mutations were not a response by the bacteria to that agent. However, in 1988, John Cairns and colleagues called into question the generality of random mutation in bacteria. Their paper and some other studies seemed to show that certain mutations occurred only (or more often) when the mutants were favored, and such mutations were called 'directed'. However, several subsequent experiments indicated that some of the evidence for directed mutation was flawed or misinterpreted, and this subject became very controversial. However, there is now widespread agreement that the most radical hypotheses put forward to explain this phenomenon – for example, a reverse flow of information from the environment through proteins and RNA back to the DNA – are incorrect.

Nonetheless, this controversy generated renewed interest in bacterial mutation, especially the mechanisms by which a cell might exercise some control over the mutational process. Attention became focused on understanding the effects of stress, such as due to starvation, on DNA repair and mutation, as well as the extent of variation among local DNA sequences in their mutability. For example, numerous studies have documented unusually high mutation rates in short repeated sequences (e.g., TTTT); the mutations are typically frameshift events involving the loss or gain of a repeated element, and they occur via the slippage of DNA strands during replication. These hypermutable sequences are not distributed randomly throughout bacterial genomes. Rather, they are found more often in genes encoding products (such as fimbriae and lipopolysaccharides on the cell surface) that are involved in pathogenicity and evasion of host immune surveillance. This distribution suggests that bacteria have evolved a simple but effective strategy to increase the mutation rate in genes that help them cope with unpredictable aspects of the environment, without inflating the load of deleterious mutations in 'housekeeping' genes whose products interact predictably with the environment. These mutations are apparently random insofar as a particular mutation does not occur as a direct response to an immediate and specific need, but they are nonrandom in their genomic distribution and may thereby promote a more favorable balance between evolutionary flexibility and conservatism.

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Exotoxins

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Defining Statement
Classification of Exotoxins
General Properties of Exotoxins
Conversion of Exotoxins into Toxoids
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Glossary

AB structure–function Structure–function organization of most bacterial exotoxins; the A domain comprises catalytic activity and the B domain comprises the receptor-binding and the translocation domains.

exotoxin A soluble protein produced by a microorganism that can enter a host cell and catalyze the covalent modification of a cellular component to alter host cell physiology.

heat-stable enterotoxins Soluble peptides that are secreted by bacteria, which bind to host cells and stimulate a signal transduction pathway within the host cell.

mechanism of action The specific reaction by which each exotoxin catalyzes to covalently modify a host cell component, such as the ADP-ribosylation of elongation factor-2 by diphtheria toxin.

N-terminal and C-terminal Bacterial toxins are often organized into distinct domains termed the amino-terminal (N-terminal) domain and the carboxy-terminal (C-terminal) domain based on their location within the primary amino acid sequence of the toxin.

pore-forming toxins Soluble proteins that are secreted by bacteria, which bind to the surface of the host cell and oligomerize to form a pore to release soluble components from the host cell.

post-translational modification A covalent modification to a cellular component that occurs subsequent to its synthesis.

proenzyme The form in which exotoxins are secreted by bacteria; these are processed to exhibit catalytic activity.

superantigens Soluble proteins that are secreted by bacteria and bind to the major histocompatibility complex of T lymphocytes. This stimulates antigen-independent proliferation of T lymphocytes.

toxoid The detoxified form of an exotoxin that is used for immunization. Conventional toxoiding is achieved with chemicals such as formalin; genetic engineering approaches have also been used to produce toxoids.

type III-secreted cytotoxins Soluble proteins that are translocated into the host cell cytoplasm via a type III secretion apparatus by host cell surface-bound bacteria. Subsequent studies identified several analogous secretion systems termed the type IV and type V secretion systems that also deliver cytotoxins from the bacterium.

vaccination The administration of an immunogen (toxoid) to stimulate an immune response that protects the host from infection by the microorganism that produces the immunogen.

Abbreviations

ARF	ADP-ribosylation factor
BT/TT	Botulinum toxin and tetanus toxin
cGMP	cyclic guanosine monophosphate
CNF	cytotoxic necrotizing factor
CTL	cytotoxic lymphocyte
EF	edema factor
ETA	exotoxin A
GM1	Ganglioside
LF	lethal factor
LT	heat-labile enterotoxin
NAD	nicotinamide adenine dinucleotide
PA	protective antigen
STa	heat-stable enterotoxin a

Defining Statement

Exotoxins are a group of soluble proteins that are secreted by the bacterium, enter host cells, and catalyze the covalent modification of a host cell component(s) to alter the host cell physiology. Both Gram-negative and Gram-positive bacteria produce exotoxins. A specific bacterial pathogen may produce a single exotoxin or multiple exotoxins. Each exotoxin possesses a unique mechanism of action, which is responsible for the elicitation of a unique pathology. Thus, the role of exotoxins in bacterial pathogenesis is unique to each exotoxin. *Corynebacterium diphtheriae* produces diphtheria toxin, which is responsible for the systemic pathology associated with diphtheria, whereas *Vibrio cholerae* produces cholera toxin, which is responsible for the diarrheal pathology associated with cholera. Exotoxins vary in their cytotoxic potency, with the clostridial neurotoxins being the most potent exotoxins of humans. Exotoxins also vary with respect to the host that can be intoxicated. Exotoxin A (ETA) of *Pseudomonas aeruginosa* can intoxicate cells from numerous species, whereas other toxins, such as diphtheria toxin, are more restricted in the species that can be intoxicated. Some bacterial toxins, such as pertussis toxin, can intoxicate numerous cell types, whereas other toxins, such as the clostridial neurotoxins, show a specific tropism and intoxicate only cells of neuronal origin. Bacterial exotoxins catalyze specific chemical modifications of host cell components, such as the ADP-ribosylation reaction catalyzed by diphtheria toxins or the deamidation reaction catalyzed by the cytotoxic necrotizing factor (CNF) produced by *Escherichia coli.* These chemical modifications may either inhibit or stimulate the normal action of the target molecule to yield a clinical pathology. Bacterial exotoxins possess an AB structure–function organization, in which the A domain represents the catalytic domain and the B domain comprises the receptor-binding domain and the translocation domain. The translocation domain is responsible for the delivery of the catalytic A domain into an intracellular compartment of the host cell.

Many bacterial exotoxins can be chemically modified to toxoids that no longer express cytotoxicity, but retain immunogenicity. Bacterial toxins can also be genetically engineered to toxoids, which may lead to a wider range of vaccine products. Exotoxins have also been used as therapeutic agents to correct various disorders, including the treatment of muscle spasms by botulinum toxin (BT). Nontoxic forms of exotoxins have been used as carriers for the delivery of heterologous molecules to elicit an immune response and as agents in the development of cell-specific chemotherapy. In addition, bacterial toxins have been used as research tools to assist in defining various eukaryotic metabolic pathways, such as G protein-mediated signal transduction.

Classification of Exotoxins

Exotoxins are soluble proteins produced by microorganisms that can enter a host cell and catalyze the covalent modification of a cellular component(s) to alter host cell physiology. The term 'host cell' refers to either vertebrate cells or cells of lower eukaryotes, such as protozoa, because some bacterial exotoxins intoxicate a broad range of host cells. The recognition that some pathogenic bacteria produced soluble components capable of producing the pathology associated with a particular disease was determined in the late nineteenth century. Roux and Yersin observed that culture filtrates of *Corynebacterium diphtheriae* were lethal in animal models and that the pathology elicited by the culture filtrate was similar to that observed during the infection by the bacterium. Subsequent studies isolated a protein, diphtheria toxin, from the toxic culture filtrates and showed that the administration of purified diphtheria toxin into animals was sufficient to elicit the pathology ascribed to diphtheria. Diphtheria toxin is a prototype exotoxin and has been used to identify many of the biochemical and molecular properties of bacterial exotoxins.

The ability of a bacterial pathogen to cause disease frequently requires the production of exotoxins, but the mere ability to produce a toxin is not sufficient to cause disease. Cholera toxin is the principal virulence factor of *Vibrio cholerae.* Administration of micrograms of purified cholera toxin to human volunteers elicits a diarrheal disease that mimics the magnitude of the natural infection. Nonetheless, nonvirulent toxin-producing strains of *V. cholerae* have been isolated and shown to lack specific biological properties, such as motility or chemotaxis. Similarly, although anthrax toxin is the principal toxic component of *Bacillus anthracis*, nonvirulent toxin-producing strains of *B. anthracis* have been isolated and shown to lack the ability to produce a polyglutamic acid capsule. An exception to this generalization is the intoxication elicited by the botulinum neurotoxins, in which ingestion of the preformed toxin is responsible for the elicitation of disease; food poisoning by botulinum neurotoxins is an intoxication rather than an infection by a toxin-producing strain of *Clostridium botulinum.*

Bacterial exotoxins are classified according to their mechanisms of action. The covalent modifications of host cell components, which are catalyzed by bacterial exotoxins, include ADP-ribosylation, deamidation, depurination, endoproteolysis, and glucosylation (**Table 1**). Most cellular targets of bacterial exotoxins are proteins, although there are exceptions such as Shiga toxin, which catalyzes the deadenylation of ribosomal RNA. In addition to exotoxins, there are several other classes of toxins that are produced by bacterial pathogens, including the pore-forming toxins, type III-secreted cytotoxins, heat-stable

Table 1 Properties of representative bacterial exotoxins

Modification	*Exotoxin*	*Bacterium*	*AB*	*Target*	*Contribution to pathogenesis*
ADP-ribosylation	Diphtheria toxin	*Corynebacterium diphtheriae*	AB	Elongation factor-2	Inhibition of protein synthesis
	Exotoxin A	*Pseudomonas aeruginosa*	AB	Elongation factor-2	Inhibition of protein synthesis
	Cholera toxin	*Vibrio cholerae*	AB5	Gsα	Inhibition of GTPase activity
	Heat-labile enterotoxin	*Escherichia coli*	AB5	Gsα	Inhibition of GTPase activity
	Pertussis toxin	*Bordetella pertussis*	AB5	Giα	Uncouple signal transduction
	C2 toxin	*Clostridium botulinum*	A–B	Actin	Actin depolymerization
	ExoS	*P. aeruginosa*	A (type III delivered)	Multiple targets including Ras	Uncoupling of Ras signal transduction
	ExoT	*P. aeruginosa*	A (type III delivered)	Crk	Uncoupling of Rac signal transduction
	SpvB	*Salmonella*	A (type III delivered)	Actin	Disruption of the actin cytoskeleton
Glucosylation	Lethal toxin	*Clostridium sordelli*	AB	Ras	Inhibition of effector interactions
	Toxin A and B	*Clostridium difficile*	AB	RhoA	Inhibition of Rho signaling
Endoprotease	Lethal toxin (anthrax toxin)	*Bacillus anthracis*	A–B	MAP kinase	Cell death
	Botulinum toxin (A–G)	*C. botulinum*	AB	SNARE proteins	Inhibition of vesicle fusion in neurons (flaccid paralysis)
	Tetanus toxin	*Clostridium tetani*	AB	Vesicle-associated membrane protein	Inhibition of vesicle fusion in neurons (spastic paralysis)
Deamidation	Cytotoxic necrotizing factor	*E. coli*	AB	RhoA	Stimulation of RhoA
Deadenylation	Shiga toxin	*Shigella* spp.	AB5	28S ribosome	Inhibition of protein synthesis
	Verotoxin	*E. coli*	AB5	28S RNA	Inhibition of protein synthesis
Adenylase cyclase	Adenylate cyclase toxin	*B. pertussis*	AB	cAMP production	Uncouple cell signaling
	Edema toxin (anthrax toxin)	*B. anthracis*	A–B	cAMP production	Uncouple cell signaling
	ExoY	*P. aeruginosa*	A (type III delivered)	cAMP production	Uncouple cell signaling
RhoGAP	SptP	*Salmonella* spp.	A (type III delivered)	Inhibition of RhoGTPase function	Inhibition of phagocytosis
	YopE	*Yersinia*	A (type III delivered)	Inhibition of RhoGTPase function	Inhibition of phagocytosis
	ExoS	*P. aeruginosa*	A (type III delivered)	Inhibition of RhoGTPase function	Inhibition of phagocytosis

enterotoxins, and superantigens. Each of these toxins fails to perform one of the properties associated with exotoxins. The pore-forming toxins are not catalytic in their action but instead disrupt cell physiology through the formation of pores in the host cell plasma membrane. The type III-secreted cytotoxins cannot enter host cells as soluble proteins but instead are injected directly into the host cell by the type III secretion apparatus of the cell-bound bacterium. The heat-stable enterotoxin and superantigens do not enter the intracellular compartment of the host cell and elicit host cell responses by triggering signal transduction pathways upon binding to the host cell membrane. In this article, initial emphasis is placed on the molecular properties of bacterial exotoxins, with a subsequent description of the general properties of pore-forming toxins, type III-secreted cytotoxins, heat-stable enterotoxins, and superantigens.

The pathology elicited by a specific exotoxin results from the catalytic covalent modification of a specific host cell component. Although diphtheria toxin and cholera toxin are both bacterial ADP-ribosylating exotoxins, the pathogenesis elicited by each exotoxin is unique. This is due to the fact that diphtheria toxin ADP-ribosylates elongation factor-2, resulting in the inhibition of protein synthesis and subsequent cell death, whereas cholera toxin ADP-ribosylates the Gs component of the heterotrimeric protein, which stimulates the activity of adenylate cyclase. The stimulation of adenylate cyclase elevates intracellular cAMP and the subsequent secretion of electrolytes and H_2O from the cell, resulting in the clinical manifestations of cholera.

General Properties of Exotoxins

Genetic Organization of Exotoxins

The genes encoding bacterial exotoxins may be located on the chromosome or located on an extrachromosomal element, such as a plasmid or a bacteriophage. Elegant experiments characterizing diphtheria toxin showed that the gene encoding this exotoxin was located within the genome of the lysogenic phage. Although both nonlysogenic and lysogenic strains of *C. diphtheriae* could establish local upper respiratory tract infection, only strains of *C. diphtheriae* lysogenized with α-phage that encoded diphtheria toxin were capable of eliciting systemic disease. This established a basic property for the pathology elicited by bacteria that produce exotoxins; bacteria establish a localized infection and subsequently produce an exotoxin, which is responsible for pathology being distanced from the site of infection.

Most exotoxins are produced only during specific stages of growth, with the molecular basis for the regulation of toxin expression varying with each bacterium. This differential expression often reflects a complex regulation of transcription, including responses to environmental conditions, such as iron. Multisubunit toxins are often organized in operons to allow the coordinate expression of their subunit components.

Secretion of Exotoxins from the Bacterium

Most bacteria secrete exotoxins across the cell membrane by the type II secretion pathway. The secretion of exotoxins by the type II secretion pathway was predicted by determining that the N-terminus of mature exotoxins had undergone proteolysis relative to the predicted amino acid sequence derived from the gene sequence encoding the exotoxin. Type II secretion is also called the general secretion pathway. Type II secretion involves the coordinate translation and secretion of a nascent polypeptide across the cell membrane. During the translation of the mRNA that encodes a type II-secreted protein, the nascent polypeptide encodes an N-terminal leader sequence that is targeted to and secreted across the cell membrane. After secretion across the cell membrane, the nascent protein folds into its native conformation and the leader sequence is cleaved by a periplasmic leader peptidase to yield a mature exotoxin.

Some Gram-negative bacteria export the assembled exotoxin from the periplasm into the external environment via a complex export apparatus. While the heat-labile enterotoxin (LT) of *Escherichia coli* remains localized within the periplasmic space, *V. cholerae* and *Bordetella pertussis* assemble their respective exotoxins, cholera toxin and pertussis toxin, within the periplasm and then transport the mature exotoxin into the external environment. Although the multiple protein components of the export apparatus have been identified, the exact mechanism for export across the outer membrane remains to be resolved.

Bacteria Produce and Secrete Exotoxins as Proenzymes

Although one property of a bacterial exotoxin is the ability to intoxicate sensitive cells, early biochemical studies observed that, *in vitro*, many bacterial exotoxins possessed little intrinsic catalytic activity. These perplexing observations were resolved by determining that bacteria produce and secrete exotoxins as proenzymes, which must be activated (processed) to express catalytic activity *in vitro*. Because exotoxins intoxicate sensitive cells, the requirements for *in vitro* activation reflect the activation steps *in vivo*. Each exotoxin requires specific conditions for activation, including proteolysis, disulfide bond reduction, or association with a nucleotide or a eukaryotic accessory protein. Some activation processes result in the release of the catalytic A domain from the B domain, whereas other activation processes appear to result in a conformational change in the catalytic A domain, rendering it catalytically active. Some exotoxins require sequential activation steps. Diphtheria toxin is activated by limited proteolysis, followed by disulfide bond reduction.

The determination of the activation mechanism of exotoxins has also provided insight into several physiological pathways of host cells. The eukaryotic protein, ADP-ribosylation factor (ARF), which activates cholera toxin *in vitro*, was subsequently shown to play a central role in vesicle formation within the eukaryotic cell. The ability of a host cell extract to activate cholera toxin is often used as a sign of the presence of ARF. Cholera toxin is activated through a series of steps where the catalytic domain of the toxin is cleaved into A1 and A2 fragments. The A1 fragment binds ARF, which activates the protein to ADP-ribosylate the alpha subunit of the heterotrimeric G_s protein to uncouple cAMP regulation within the host cell. Characterization of the mechanisms that pertussis toxin and cholera toxin use to intoxicate eukaryotic cells has provided insight into the pathways for eukaryotic G protein-mediated signal transduction. The ability of pertusis toxin to inhibit the action of a ligand in a signal transduction pathway is often used to implicate a role for G proteins in that signaling pathway.

AB Structure–Function Properties of Exotoxins

Most bacterial exotoxins possess AB structure–function properties (**Figure 1**). The A domain is the catalytic domain, whereas the B domain includes the translocation and binding domains of the exotoxin. Exotoxins are organized into one of several general types of AB organization. The simplest AB organization is represented by diphtheria toxin, in which the A domain and B domain are contained in a single protein. Diphtheria toxin is the prototype for this class of AB exotoxin. Diphtheria toxin is a 535-amino acid protein in which the N-terminus constitutes the ADP-ribosyltransferase domain and the C-terminus comprises the translocation domain and receptor-binding domain. The AB5 exotoxins are composed of six proteins that are noncovalently associated as an oligomer. Cholera toxin is the prototype for the AB5 exotoxin. The A domain of cholera toxin constitutes the ADP-ribosyltransferase domain, whereas the B5 domain is composed of five identical proteins, forming a pentamer. This is organized into a ring structure, on which the A domain is positioned. The five proteins that make up the B domain may be identical, as is the case for cholera toxin and the LT of *E. coli*, or may be different proteins that form a nonsymmetrical ring structure, as observed with the B oligomer of pertussis toxin.

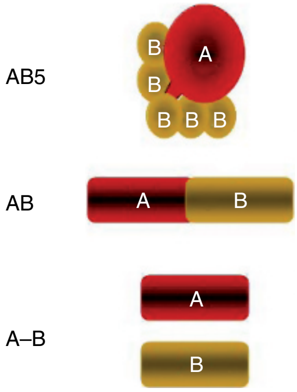

Figure 1 Bacterial exotoxins possess AB structure–function organization. There are three general AB organizations of bacterial exotoxins. The A domain (red) represents the catalytic domain whereas the B domain comprises the translocation and receptor-binding domains (brown). AB5 is represented by cholera toxin of *Vibrio cholerae*, which is composed of six noncovalently associated proteins. AB is represented by diphtheria toxin of *Corynebacterium diphtheriae*, in which the A domain and the B domain are included in a single protein. A–B is represented by anthrax toxin of *Bacillus anthracis*, which is composed of two nonassociated proteins; the two proteins associate after the binding and processing of the B component on the host cell membrane.

The third class of AB exotoxin is composed of proteins that are not associated in solution, but associate following the binding and processing of the B domain to the host cell. C2 toxin is an example of this class of A–B exotoxin. C2 toxin is a bipartile exotoxin composed of a protein that encodes the catalytic A domain and a separate protein that encodes the B domain. The A domain C2 toxin ADP-ribosylates actin. The B domain protein of C2 binds to sensitive cells and is nicked by a eukaryotic protease. The processed B components oligomerize and are then capable of binding either of the A domain proteins. Anthrax toxin is a major virulence factor of *B. anthracis*. Like the C2 toxin, anthrax toxin is organized as an AB exotoxin that is composed of two unique A domains (edema factor (EF), an adenylate cyclase; lethal factor (LF), a zinc protease) and a B domain (termed the protective antigen (PA)), which are not associated in solution. The A domains associate with the B domain subsequent to the binding and the oligomerization of the B domain on the surface of sensitive host cells. Recent studies observed that three A domains can bind to each heptameric B domain on the host cell surface.

Although the A domain possesses the catalytic activity of the exotoxin, the B domain possesses two specific functions, receptor binding and translocation capacity. Each exotoxin uses a unique host cell surface component as a receptor. The cell surface receptor for each exotoxin may be specific. The cell surface receptor for cholera toxin is the ganglioside (GM1) whereas diphtheria toxin binds directly to the epidermal growth factor precursor. In contrast, the binding of pertussis toxin appears to be less specific, as pertussis toxin is able to bind numerous cell surface proteins. The ability to bind its cell surface receptor is an absolute requirement for an exotoxin to intoxicate a host cell because the deletion of the receptor-binding domain renders the exotoxin essentially noncytotoxic. After binding to the cell surface, some exotoxins are proteolytically processed or are processed during endocytic vesicle transport.

The second function of the B domain includes translocation capacity, which is responsible for the delivery of the A domain across the cell membrane. The presence of a translocation domain was predicted from early structure–function studies of diphtheria toxin, which showed that in addition to the catalytic domain and receptor-binding domain, a third function was required for the efficient expression of cytotoxicity. This third function was subsequently shown to correspond to a region of diphtheria toxin that had the propensity to interact with membranes. The crystal structure of diphtheria toxin revealed the presence of three distinct domains, representing the catalytic, translocation, and receptor-binding functions.

Exotoxins Enter Host Cells via Distinct Pathways

Although A domain translocation is one of the least understood aspects of the intoxication process of exotoxins, there are several general themes that are involved in translocation of the A domain across the cell membrane. One translocation mechanism uses a pH gradient within the endosome to stimulate protein conformational changes in the B domain, making it competent to interact with the endocytic vesicle. After insertion into the endocytic membrane, the B domain generates a pore that is believed to be involved in the translocation of the A domain across the vesicle membrane in an unfolded form. After the translocation across the endocytic membrane, the A domain refolds to its native conformation. Subsequent to translocation of the A domain across the vesicle membrane, glutathione may reduce the disulfide that connects the A domain with the B domain and release the A domain into the cytoplasm. The potency and catalytic potential of exotoxins was demonstrated by the observation that the introduction of one molecule of the catalytic domain of diphtheria toxin into the intracellular cytoplasm was sufficient to inhibit host cell physiology, resulting in cell death.

Other toxins, such as cholera toxin and exotoxin A (ETA) of *Pseudomonas aeruginosa*, appear to use retrograde transport to enter the interior regions of the cell. Movement appears to occur through retrograde transport from the endosome to the Golgi apparatus and ultimately to the endoplasmic reticulum. Many exotoxins that are ultimately delivered to the endoplasmic reticulum possess a KDEL (Lys-Asp-Glu-Leu)-like retention signal sequence on their C-terminus. Although the details for the actual transport pathway remain to be determined, studies with chimeric proteins have shown that the introduction of a KDEL retention sequence is sufficient to retrograde transport a protein, which is normally delivered only to the early endosome, into the endoplasmic reticulum. Thus, there is physiological precedence for the use of the KDEL sequence to retrograde transport exotoxins toward the endoplasmic reticulum. One of the basic questions concerning the intoxication process of these exotoxins is the actual mechanism of translocation and whether or not eukaryotic proteins assist in the translocation process. Recent studies on the translocation process of anthrax toxin support the direct translocation of the A domain through a stable, gated channel formed by the heptameric B domain.

Covalent Modification of Host Cell Components by Exotoxins

Exotoxins use several unique mechanisms to covalently modify host cell components. The major classes of reactions are the covalent addition of a chemical group to the target protein, the cleavage of a chemical group from a target protein, and the endoproteolytic cleavage of a peptide bond of the target protein.

The ADP-ribosylation of host proteins is the prototype mechanism of action of bacterial exotoxins. Numerous bacterial exotoxins catalyze the ADP-ribosylation of specific host proteins and elicit physiological changes. In the ADP-ribosylation reaction, exotoxins use the oxidized form of nicotinamide adenine dinucleotide (NAD) as the substrate and transfer the ADP-ribose portion of NAD to a specific amino acid via an *N*-glycosidic linkage of ADP-ribose onto the host target protein. The specific type of amino acid that is ADP-ribosylated within the target protein varies with the specific exotoxin. ADP-ribosylation may either inactivate or stimulate the activity of the target protein. Diphtheria toxin ADP-ribosylates elongation factor-2 on a post-translationally modified histidine residue called diphthamide. ADP-ribosylated elongation factor-2 is unable to perform its translocation of nascent polypeptides in the ribosome, which results in the inhibition of protein synthesis and subsequent cell death. In contrast, cholera toxin ADP-ribosylates the Gs component of a heterotrimeric G protein. ADP-ribosylated Gs is locked in an active conformation, which results in the stimulation of adenylate cyclase and the subsequent elevation of intracellular cAMP. Likewise, deamidation of Gln63 in RhoA by *E. coli* cytotoxic necrotizing factor (CNF) results in a constitutively active RhoA protein. Note that although most host targets for exotoxins are proteins, Shiga toxin catalyzes the deadenylation of a specific adenine on 28S RNA.

Recall that each exotoxin modifies a specific host cell component, which is responsible for the specific pathology elicited by that exotoxin. Although there are no absolute rules for the types of proteins targeted for covalent modification, the most frequent targets are the nucleotide-binding proteins that are involved in signal transduction pathways, including both the heterotrimeric G proteins and the small molecular weight GTP-binding proteins of the Ras superfamily. It is not clear whether this class of host protein is targeted for modification due to the presence of a common structural motif or due to its critical role in host cell metabolism.

Molecular and Structural Properties of Bacterial Exotoxins

Early biochemical studies provided significant advances in defining the structure–function properties of exotoxins, resolving many of the exotoxin mechanisms of action and developing the concept that exotoxins have AB organization. Molecular genetics and structural biology have extended earlier studies and provided a more detailed understanding of the biochemical and molecular relationships among the exotoxins. The biochemical characterization of diphtheria toxin and ETA of *P. aeruginosa* showed that these two exotoxins catalyzed kinetically identical reactions during the ADP-ribosylation of elongation factor-2. In addition, both diphtheria toxin and ETA were shown to possess an active site glutamic acid, which was subsequently shown to be a signature property of exotoxins that catalyze the ADP-ribosyltransferase reaction. These observations predicted that ADP-ribosylating exotoxins would possess considerable primary amino acid homology. Thus, the determination that the genes encoding diphtheria toxin and ETA shared little primary amino acid homology was unexpected. This paradox was resolved after the analysis of the three-dimensional structures of ETA and the LT of *E. coli* and subsequently confirmed with diphtheria toxin. The three-dimensional structures of ETA and LT showed little similarity in their respective receptor-binding domains and translocation domains; however, the catalytic domains of ETA and LT, which are composed of seven discontinuous regions of each protein, could be superimposed on each other despite possessing homology at only 3 of the 43 amino acids. One of the homologous amino acids in ETA and LT was the signature active site glutamic acid. This was a remarkable finding because ETA and LT ADP-ribosylate different host target proteins and possess different AB organization. A common theme has evolved for describing the structure–function properties of this family of bacterial exotoxins in which the ADP-ribosylating exotoxins possess a conserved three-dimensional structure in their active sites, despite the lack of primary amino acid homology. These findings have provided a framework for the study of other classes of exotoxins produced by divergent groups of bacteria.

Conversion of Exotoxins into Toxoids

Chemical Detoxification of Bacterial Exotoxins

Shortly after the determination that toxic components were associated with bacterial pathogens, several studies showed that cell extracts or cell cultures of a pathogen could be treated with chemical denaturants, such as formalin, to produce nontoxic immunogenic material that could prevent disease upon subsequent exposure to that pathogen. In the case of diphtheria toxin and tetanus toxin (TT), chemical modification with formalin produced toxoids that were used as acellular vaccines in large-scale immunizations. This resulted in a remarkable decrease in the incidence of both diphtheria and tetanus within the populations that were immunized. In areas where these toxoids are not administered, diphtheria and tetanus remain clinically important diseases. In addition to formalin, other chemicals have been used to detoxify bacterial exotoxins, including glutaraldehyde and hydrogen peroxide. In contrast, the chemical toxoiding of other exotoxins, such as cholera toxin and pertussis toxin, has been more difficult because the treatment of these toxins with denaturants often results in a reduction of immunogenicity. Thus, there is a need to develop alternative strategies, such as genetically engineered and subunit vaccines, to eliminate the cytotoxicity of certain exotoxins without compromising their immunogenicity.

Genetic Detoxification and Subunit Vaccines of Bacterial Exotoxins

Developments in genetic engineering have provided an opportunity to produce recombinant forms of bacterial exotoxins that possess greatly reduced toxicity, but retain immunogenicity. The use of genetic engineering to develop a toxoid of pertussis toxin has been successful. The whole-cell pertussis vaccine is composed of a chemically treated preparation of *B. pertussis*, which is effective in the elicitation of a protective immune response after mass immunization. However, the whole-cell pertussis vaccine is acutely reactive when administered to children because of the crude nature of the vaccine that includes endotoxin. Recently, a component pertussis vaccine has been developed that is administered with diphtheria toxoid and tetanus toxoid (termed the DTPa vaccine), which produces fewer adverse reactions in children than the whole-cell pertussis vaccine. The reactivity of the acellular pertussis vaccine is so low that administration of this vaccine in adults is nearing approval, an important development as adults are considered a primary carrier of *B. pertussis*. Pertussis toxin, a primary virulence determinant of *B. pertussis*, is an exotoxin that ADP-ribosylates the Gi component of heterotrimeric G proteins and effectively uncouples signal transduction between the G protein-coupled receptor and the G protein.

Genetically engineered forms of pertussis toxin have been produced that possess essentially no catalytic activity or cytotoxicity, but that maintain native conformation and elicit a protective immune response when used as an immunogen. These recombinant noncytotoxic forms of pertussis toxin have been engineered with multiple mutations in their active site, virtually eliminating the risk of reversion to a cytotoxic form. Similar strategies are being applied to other bacterial exotoxins with the goal of engineering acellular vaccine candidates.

Subunit vaccines represent another approach to developing safe and efficient vaccines. This strategy implies that a domain of the exotoxin can be identified that elicits a protective immune response against exotoxin challenge in the host. One example of vaccination is the development of a subunit vaccine against botulism. Botulism is a toxin-mediated disease elicited by botulinum toxin (BT). BT is the most toxic protein for humans and is an AB toxin (**Figure 2**). Recent studies have shown that immunization with the C-terminal portion of the B domain stimulates a protective immune response against challenge by BT. Recombinant forms of this C-terminal B domain have been produced in yeast and *E. coli* and represent the next generation of botulism vaccine.

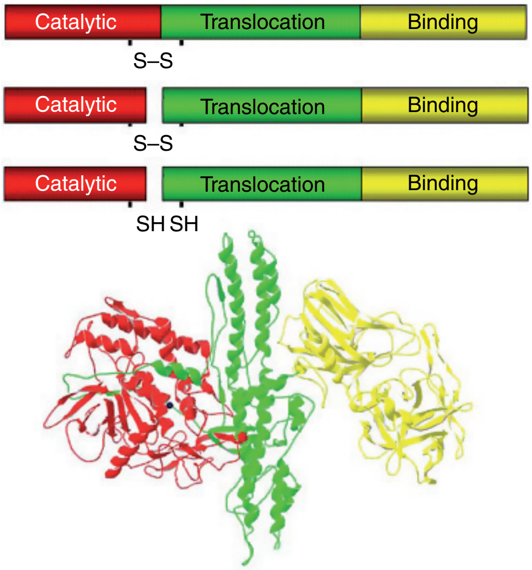

Figure 2 Bacterial exotoxins are produced as proenzymes. upper panel – Most bacterial exotoxins are produced as proenzymes that undergo processing to express catalytic activity. The sequential processing of botulinum toxin involves protein cleavage between the catalytic A domain (red) and the translocation (green) and receptor-binding (yellow) B domain. The A and B domains are connected by a disulfide bond, which is reduced as the A domain is translocated into the host cytoplasm by agents such as reduced glutathione. Lower panel – A ribbon diagram of botulinum neurotoxin is shown (Protein Data Bank No. 3BTA). The catalytic A domain (red) is linked to the translocation (green)/receptor-binding (yellow) B domain.

Therapeutic Applications of Exotoxins

One of the most exciting areas of bacterial exotoxin research has been the development of strategies to use exotoxins in therapeutic disciplines. Some therapies use the native cytotoxic form of the exotoxin. Other therapies use either the A or B domain, which is conjugated to a heterologous binding component or to effector elements, respectively, to produce a chimeric molecule with directed properties.

Botulinum toxin and tetanus toxin (BT/TT) are each a single protein that is organized as an AB exotoxin. The N-terminus of BT/TT expresses endoprotease activity within neurons, which results in the paralysis associated with botulism and tetanus, and constitutes the A domain, whereas the B domain possesses neuron-specific receptor-binding activity. The specific association of the B domain with neuronal cells is responsible for the clinical manifestation of these neurotoxins. BT/TT appear to enter neuronal cells by receptor-mediated endocytosis and deliver the A domain to the cytosol, where the A domain catalyzes the endoproteolytic cleavage of host SNARE proteins that are involved in vesicle fusion. BT can be introduced into the muscles surrounding the eye to temporarily reduce muscle spasms associated with several clinical disorders, such as blepharospasm, an involuntary contraction of eye muscles. This application for BT has been expanded and this toxin represents one of the most widely used therapeutic agents in clinical medicine. In contrast, the extreme potency of BT to humans has made this a potential agent for malicious application.

Diphtheria toxin has been used as a carrier to stimulate an immune response against several epitopes. One epitope is polyribitolphosphate, a component of the polysaccharide capsule of *Haemophilus influenzae* type b (Hib). Early attempts to elicit an effective immune response to purified Hib antigen resulted in the production of a T-cell-independent immune response that did not yield an effective memory. A noncatalytic mutant of diphtheria toxin, CRM197, has been used as a carrier for the Hib epitope. Immunization with the CRM197–Hib conjugate yielded a strong T-dependent immune response. Mass immunization with Hib conjugates has resulted in a dramatic reduction in the number of cases of Hib in the immunized population.

Due to their potency, the catalytic A domain of exotoxins have been used in the construction of chimeric immunotoxins that are designed to target cancer cells. Early studies used conjugates that were composed of the A domain of the diphtheria toxin coupled to an antibody that recognized a cell surface-specific antigen. The A chain of the diphtheria toxin was used in the first generation of immunotoxins because it was shown to possess impressive cytotoxic potential when introduced into the cytosol of eukaryotic cells. Introduction of a single molecule of the A chain of diphtheria toxin into the cytosol is sufficient to kill that cell. In cell culture, these chimeras have proven to be both potent and antigen specific.

Anthrax toxin is a bipartite toxin composed of two nonassociated proteins. The B component of anthrax toxin, termed PA, binds sensitive cells, where PA is processed and undergoes oligomerization to form a heptameric structure on the cell surface. The PA heptamer can bind either of two A domains that are secreted from *B. anthracis* independent of PA, LF, or EF. These A–B toxin complexes enter cells via receptor-mediated endocytosis, and the A domain is translocated into the host cell cytosol upon acidification in the early endosome. A truncated, noncytotoxic form of LF has been used to deliver epitopes into antigen-presenting cells to elicit a cytotoxic lymphocyte (CTL) response. In this nontoxic anthrax delivery system, PA is added to antigen-presenting cells with a nontoxic LF–CTL epitope chimera for antigen presentation. One of the more attractive aspects of this CTL epitope delivery system is that small amounts of PA are required to present antigen. Ongoing research involves the determination of clinical situations for the use of these chimeras in a therapeutic arena.

Other Classes of Bacterial Toxins

Pore-Forming Toxins

The lack of a catalytic A domain differentiates the pore-forming toxins from exotoxins. Thus, the pathology associated with pore-forming toxins is due solely to the generation of a pore within the membrane of the host cell. Several bacterial pathogens produce pore-forming toxins, some of which are secreted by a type I secretion pathway. Unlike type II-secreted proteins, the N-terminus of type I-secreted proteins is not processed. Type I-secreted proteins possess a polyglycine signal sequence in the C-terminus of the mature toxin. There are several classes of pore-forming toxins, including members of the hemolysin family of pore-forming toxins, the aerolysin family of pore-forming toxins, and the α-toxin of *Staphylococcus aureus*. Host cell specificity differs among pore-forming toxins. The crystal structures of several of the pore-forming toxins have been determined. The molecular events generating a pore in the membrane of a host cell have been proposed for the aerolysin family of pore-forming toxins. Aerolysin is exported by *Aeromonas hydrophilia* as a monomeric molecule, which binds to the host cell. The monomer is proteolytically processed and subsequently undergoes oligomerization. The oligomerized complex is inserted into the membrane and generates a pore in the center of the complex, causing the release of the cytoplasmic components of the host cell.

Type III-Secreted Cytotoxins

The lack of a B domain differentiates the type III-secreted cytotoxins from exotoxins. Thus, the organization of the type III-secreted cytotoxins may be represented as A domains that are specific effector proteins. Type III-secreted cytotoxins are transported directly into the host cells by cell surface-bound bacteria. Type III secretion of bacterial proteins is a recently defined pathway for the delivery of proteins into the cytoplasm of host cells. Type III-secreted proteins were initially recognized by the fact that the secreted mature cytotoxins were unique to proteins secreted by either the type I or the II secretion pathways, whereas the N-terminus of type III-secreted proteins is not processed nor is there a polyglycine motif in their C-terminus. Although it is clear that a complete type III secretion apparatus is required for the transport of type III-secreted proteins into the host cytoplasm, the mechanism for the delivery of type III-secreted proteins across the host cell membrane remains to be resolved. Numerous bacteria have been shown to possess type III secretion pathways, including members of the genera *Escherichia, Pseudomonas, Shigella, Salmonella*, and *Yersinia*. Cytotoxicity elicited by type III-secreted cytotoxins has an absolute requirement for the type III secretion apparatus of the bacterium, as purified forms of the cytotoxins are not toxic to host cells.

The A domains of type III-secreted cytotoxins catalyze several unique mechanisms of action, including the depolymerization of the actin cytoskeleton, phosphatase activity, ADP-ribosyltransferase activity, and the stimulation of apoptosis. Each type III secretion apparatus appears capable of delivering numerous type III-secreted proteins into the host cell. Recent studies have identified additional secretion systems that use bacteria to translocate proteins across the bacterial cell envelope. The type IV secretion system utilizes an apparatus that is evolutionarily related to the apparatus used by bacteria for DNA transfer between bacteria. The type IV secretion apparatus has several applications: In *B. pertussis*, type IV secretion is used to transfer pertussis toxin from the periplasm across the cell envelope into the extracellular environment, whereas in *Agrobacterium tumefaciens*, the type IV secretion system is used to inject effector molecules directly into the host cells. The type V secretion system is an autotransporter system in which a protein is composed of a catalytic domain that is transported across the bacterial cell envelope by another component of the protein termed the autotransporter domain. There are numerous type V-secreted proteins with VacA, a virulence factor of *Helicobacter pylori*, one of the most noted examples of a type V-secreted protein.

Heat-Stable Enterotoxins

The inability of the heat-stable enterotoxins to enter the host cell or possess catalytic activity differentiates the heat-stable enterotoxins from exotoxins. Several genera of bacteria produce heat-stable enterotoxins, including *Escherichia* and *Yersinia*. The heat-stable enterotoxin a (STa) of *E. coli* is the prototype toxin of this group. *E. coli* secretes STa into the periplasm as a 72-amino acid precursor in which three intramolecular disulfide bonds are formed and processed into a 53-amino acid form. The 53-amino acid form of STa is exported into the environment where a second proteolytic cleavage results in the production of an 18- or 19-amino acid mature STa molecule. The mature STa binds to a protein receptor on the surface of epithelial cells, which results in an increase in the intracellular concentrations of cyclic guanosine monophosphate (cGMP). The intracellular increase in cGMP results in a stimulation of chloride secretion and net fluid secretion, resulting in diarrhea.

Superantigens

The inability of superantigens to enter the host cell or possess catalytic activity differentiates the superantigens from exotoxins. Superantigens are soluble proteins of approximately 30 kDa that are secreted by bacteria that possess mitogenic properties. Superantigens are produced by both *Streptococcus* and *Staphylococcus*. The superantigens bind to a component of the major histocompatibility complex of T lymphocytes through an antigen-independent mechanism, which stimulates proliferation of a large subset of T lymphocytes.

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Extremophiles: Acidic Environments

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Further Reading

Glossary

autotroph An organism that obtains its carbon by fixing carbon dioxide, bicarbonate, or other C_1 compound.

chemolithotroph A prokaryote that uses an inorganic energy source and fixes carbon dioxide.

FISH (fluorescent* in situ *hybridization) A technique for identifying individual cells using molecular (RNA) probes.

heterotroph An organism that obtains both its carbon and energy from an organic source.

lentic waters Nonflowing continental waters, such as ponds and lakes.

lotic waters Flowing continental waters, such as streams and rivers.

mixotroph A prokaryote that uses an inorganic energy source and an organic carbon source.

RISCs (reduced inorganic sulfur compounds) Oxysulfur anions other than sulfate.

snotites Small gelatinous growths of microorganisms that grow suspended from the roofs of underground mines and caves.

sulfidogen An organism that generates (hydrogen) sulfide.

Abbreviations

AMD	acid mine drainage
DGGE	denaturing gradient gel electrophoresis
FISH	fluorescent *in situ* hybridization
PGM	platinum group metal
RISC	reduced inorganic sulfur compound
SRB	sulfate-reducing bacteria

Defining Statement

This article gives an overview of the nature of extremely acidic environments and of the biodiversity of microorganisms found within them. Ways in which acidophiles interact with each other in both positive and negative fashions are described. Finally, the microbial ecology of some of the most widely studied extremely acidic environments on our planet is discussed.

Nature and Origin of Extremely Acidic Environments

Oceanic waters, which constitute the largest biome on planet Earth, are uniformly moderately alkaline (pH 8.2–8.4). In contrast, some lentic and lotic waters, soils, and anthropogenic environments are moderately acidic (pH 3–5) or extremely acidic ($\text{pH} < 3$). In some rare cases, environments that have recorded negative pH values have been documented. Living organisms that are active in extremely acidic environments are now known to be far more diverse than was recognized even a couple of decades ago. As with other extremophiles, acidophiles tend to be specialized life-forms, in that many are unable to grow in neutral pH environments. The majority of acidophiles are prokaryotic microorganisms, and these comprise a large variety of phylogenetically diverse Bacteria and Archaea, though some single-celled and multicellular eukaryotes are known to grow in highly acidic ponds and streams.

Extremely acidic environments may be formed by processes that are entirely natural, though human activities have became increasingly important in generating such sites. While the scale of human impact has paralleled global industrialization, small-scale anthropogenic generation of acidic, metal-polluted environments probably began in the Bronze Age. Overall, the majority of extremely acidic sites that now exist on planet Earth are associated with one particular human activity – the mining of metals and coal.

There are a number of important (in terms of their scale) microbial activities that can generate acidity. Among the most important of these is the formation of organic acids as waste products in either anaerobic (fermentative) or aerobic metabolisms. However, the generation of strong inorganic acids by aerobic microorganisms gives rise to the most acidic environments on our planet. Nitrification (the formation of nitric acid from ammonium) is potentially one of these, though the process is self-limiting in poorly buffered environments, as the majority of nitrifying bacteria are highly sensitive to even mild acidity. In contrast, prokaryotes that oxidize sulfur (in many of the large variety of reduced forms of this element that exist) include species that grow in neutral pH and moderately alkaline environments, as well as those that grow optimally in acidic environments. Indeed, some of the most extremely acidophilic life-forms known are those that obtain energy by oxidizing reduced sulfur to sulfuric acid.

Geothermal Areas

Elemental sulfur may occur in geothermal areas (e.g., around the margins of fumaroles) where it can form by the condensation of sulfur dioxide and hydrogen sulfide, two common volcanic gases (eqn [1]):

$$SO_2 + 2H_2S \rightarrow 2H_2O + 3S^0 \qquad (1)$$

Oxidation of sulfur by acidophilic bacteria and archaea generates sulfuric acid (eqn [2]):

$$S^0 + H_2O + 1.5O_2 \rightarrow H_2SO_4 \qquad (2)$$

This can result in severe acidification of environments both on the micro- (i.e., microbial habitats) and on the macroscale. Oxidation of sulfide minerals (see 'Mine-impacted environments') may also contribute to acid genesis in these locations. Whether or not specific sites develop net acidity depends on how effectively acid generation is counterbalanced by the dissolution of basic minerals, such as carbonates. Geothermal, sulfur-rich, acidic sites are known as solfatara (**Figure 1**); water temperatures in solfatara fields approach boiling point (~85–100 °C, depending on altitude) but tend to cool rapidly as the water flows from the source of the geothermal spring. These sites may therefore be colonized by a variety of acidophilic microorganisms that have different temperature optima, and are therefore very fertile locations for isolating novel acidophilic microorganisms.

High-temperature environments that host solfatara and acid streams occur in zones of volcanism and in areas where the earth's crust is relatively thin. Examples of terrestrial and shallow marine locations include Yellowstone National Park (USA); Whakarewarewa (New Zealand); Krisuvik (Iceland); the Kamchatka Peninsula (Russia); Sao Michel (Azores); Volcano, Naples, and Ischia (all Italy); Djibouti (Africa); and some Caribbean islands, such as Montserrat and St. Lucia. Related to these are deep and abyssal submarine hydrothermal systems, such as the Mid-Atlantic Ridge, the East Pacific Rise, the Guaymas Basin, and active seamounts (e.g., around Tahiti). In contrast to many terrestrial sites, submarine hydrothermal systems are generally in the range pH 3–8, and saline, due to the high buffering capacity of seawater.

Mine-Impacted Environments

Many of the most important base metals (such as copper, lead, and zinc) used by humankind are sourced mostly from sulfide minerals. In addition, many precious metals including gold, silver, and PGMs (platinum group metals) are

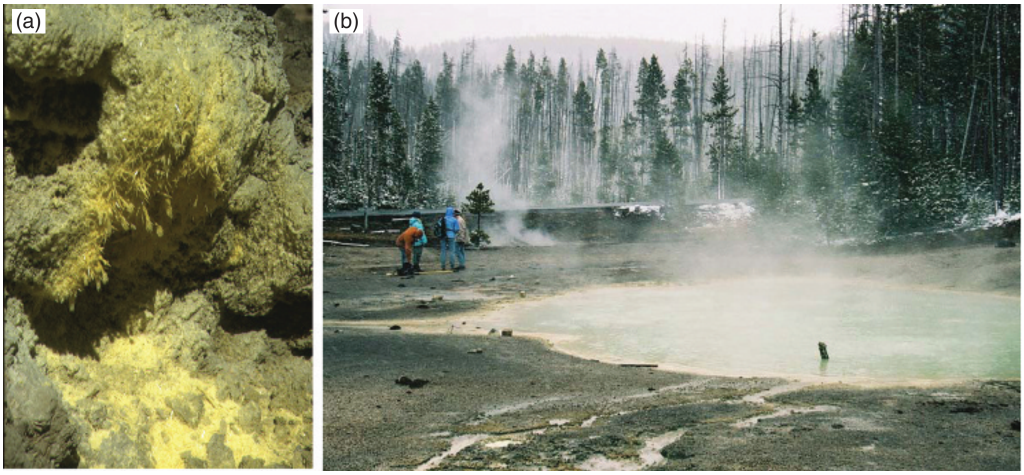

Figure 1 (a) Elemental sulfur forming from gases venting the Soufriere Hills volcano on Montserrat, West Indies; (b) an acidic geothermal pool (Frying Pan Hot Spring) in Yellowstone National Park, Wyoming.

often found in association with sulfidic ores. Mining of metallic ores has, in the past, involved smelting whole rocks, though the advent of concentration techniques (mostly involving froth flotation and separation of target minerals) and nonpyrometallurgical techniques (such as pressure oxidation and biological processing) have had, and continue to have, a major impact on the mining industry. Production of mineral concentrates results in the generation of large quantities of waste minerals that, because of the intensive rock grinding involved, are fine grain. These waste minerals (referred to as tailings) are usually disposed of in large lagoons, which may, in time, become drained thereby allowing ingress of oxygen and dissolution of the minerals, a process often paralleled by intensive acidification. Apart from this, waste rocks from mines and the abandoned mines themselves can serve as source points for the generation of metal-rich, acidic wastewaters.

The mining of copper ores, which is carried out in many different parts of the world, is one potential source of acid pollution. Copper exists in a variety of sulfide minerals, of which the most important (quantitatively) is the mixed copper–iron sulfide chalcopyrite (generally notated as $CuFeS_2$, though a more accurate mineral formulation is probably $CuFeS_{1.5}$, as both copper and iron occur in their more reduced ionic forms). Other significant copper minerals include single-metal sulfides (chalcocite, Cu_2S; and covellite, CuS) and mixed-metal sulfides bornite (Cu_5FeS_4) and enargite (Cu_3AsS_4). The sulfide moiety in these minerals represents a source of energy that can be utilized by some lithotrophic (literally, rock-eating) prokaryotes, many of which are obligate acidophiles (**Figure 2**). In addition, the ferrous iron present in chalcopyrite and bornite is a second potential energy source for mineral-oxidizing Bacteria and Archaea. These microorganisms require both oxygen and water (though little else) to facilitate their attack on the minerals, which is why mine wastes may be safely stored in environments that are either totally dry or anoxic. In moist, aerated environments, however, the minerals are prone to oxidative dissolution, resulting in the release and potential solubilization of their component metals.

Acid genesis, however, is relatively limited when copper sulfides are (biologically) dissolved. In contrast, the iron disulfide mineral pyrite (FeS_2; 'fool's gold'), which is the most abundant sulfide mineral in the lithosphere and which is invariably associated (often as the dominant mineral) with copper and other metal sulfide ores, generates significant levels of acidity due to its greater sulfur content. Pyrite has served as the model mineral for most studies of microbial attack on sulfide minerals and, although there have been various schemes proposed, that described by Wolfgang Sand of Duisburg–Essen University is generally regarded as the most accurate. In this, the initial attack on the hard, dense mineral is by ferric iron, which is a powerful oxidizing agent in acidic liquors. Ferric iron oxidizes the sulfur moiety of the mineral to thiosulfate, and in so doing is reduced to ferrous iron (eqn [3]):

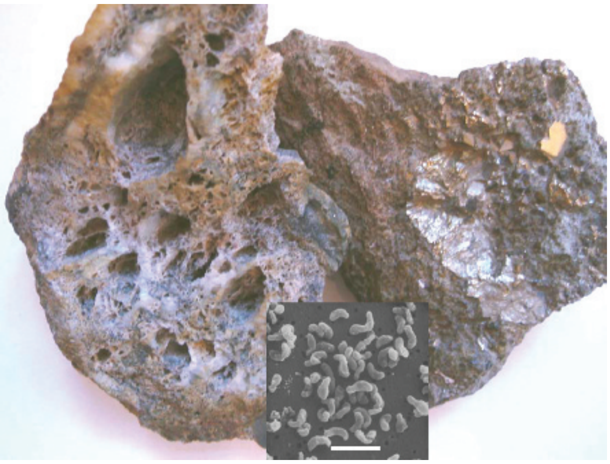

Figure 2 Partial dissolution of a sulfidic rock by chemolithotrophic bacteria. The rock on the right is freshly exposed and contains grain of pyrite (fool's gold) and other metal sulfides. That on the left has been exposed to attack by chemolithotrophic acidophiles, and the sulfide minerals have been effectively dissolved, leaving a porous remnant composed of inert minerals. Inset: the mineral-oxidizing acidophile *Leptospirillum ferrooxidans* (the scale bar represents 2 μm).

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + S_2O_3{}^{2-} + 6H^+ \qquad (3)$$

The ferrous iron formed is reoxidized to ferric iron by a variety of iron-oxidizing acidophilic bacteria and archaea in an oxygen-consuming reaction (eqn [4]):

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \qquad (4)$$

Thiosulfate is unstable in acidic liquors (particularly when ferric iron is present) and oxidizes to form a variety of other reduced inorganic sulfur compounds (RISCs) such as trithionate ($S_3O_6{}^{2-}$) and tetrathionate ($S_4O_6{}^{2-}$), as well as elemental sulfur (S^0). The latter can all serve as substrates for sulfur-oxidizing Bacteria and Archaea and are oxidized, when oxygen is available, to sulfuric acid, thereby generating the extreme acidity that helps maintain a suitable pH required by the mineral-oxidizing microorganisms.

A second mechanism has been proposed for minerals, such as sphalerite (ZnS) and galena (PbS), that are soluble in sulfuric acid. In this scenario (the polysulfide mechanism) the metal–sulfur bond is broken by proton attack and hydrogen sulfide (H_2S) is liberated. If ferric iron is also present, concomitant attack by iron and protons results in the proposed formation of H_2S^+, which dimerizes to form free disulfide (H_2S_2), and is further oxidized forming, ultimately, elemental sulfur. In the absence of sulfur-oxidizing prokaryotes, this sulfur accumulates, and though in their presence it is oxidized to sulfuric acid.

Dissolution of sulfidic ores not only produces acidity, but also generates liquors that contain concentrations of base metals (copper, zinc, manganese, etc.) and aluminum that are far greater than those found in most surface waters. The two reasons for this are (1) the occurrence of these metals in sulfide minerals and others (many aluminosilicates) that spontaneously degrade at low pH, and (2) the far greater solubility of these metals in low pH than in circumneutral pH solutions. Exceptions to the latter are metals, such as molybdenum and vanadium, that occur mostly as oxyanions, rather than cations. Metalloids, most significantly arsenic, can also be present at highly elevated concentrations in sulfide ore leach liquors; arsenopyrite (FeAsS) and realgar (As_4S_4) are two other relatively common sulfide minerals. Waters percolating through fissures in worked-out underground mines, as well as those draining stockpiled mine waste rock dumps and mine tailings, become enriched in these soluble metals and metalloids. At their point of discharge from underground mines or tailings ponds, mine waters are frequently devoid of oxygen and appear untainted. However, flowing waters become increasingly aerated, facilitating the oxidation of uncolored ferrous iron (usually the dominant dissolved metal found in mine drainage waters) to highly colored (yellow-red) ferric iron. This is why mine water-impacted waters are, in the main, very obvious sites of water pollution. Depending on pH, the ferric iron formed will either remain in solution or hydrolyze (react with water) to produce a variety of solid phase minerals (e.g., schwertmannite ($Fe_8O_8(OH)_6SO_4$), ferrihydrite ($5Fe_2O_3.9H_2O$), and amorphous ferric hydroxide ($Fe(OH)_3$)):

$$Fe^{3+} + H_2O \rightarrow Fe(OH)_3 + 3H^+ \qquad (5)$$

This has two important consequences: First, the reaction generates protons, as illustrated in eqn [5], thereby helping to maintain the acidity of water. Aluminum and manganese also behave similarly, but these metals are generally less abundant than iron in mine waters. Second, the precipitates that form sink to the bottom of the stream, forming a dense coating (known in Europe as ochre and in the USA as yellow boy) that can seriously impact benthic life. In the most extremely acidic mine waters (pH < 2.5), ferric iron remains in solution and the resulting red water color is often reflected in the names given to these streams or rivers, most famously, the Rio Tinto in Spain (**Figure 3**).

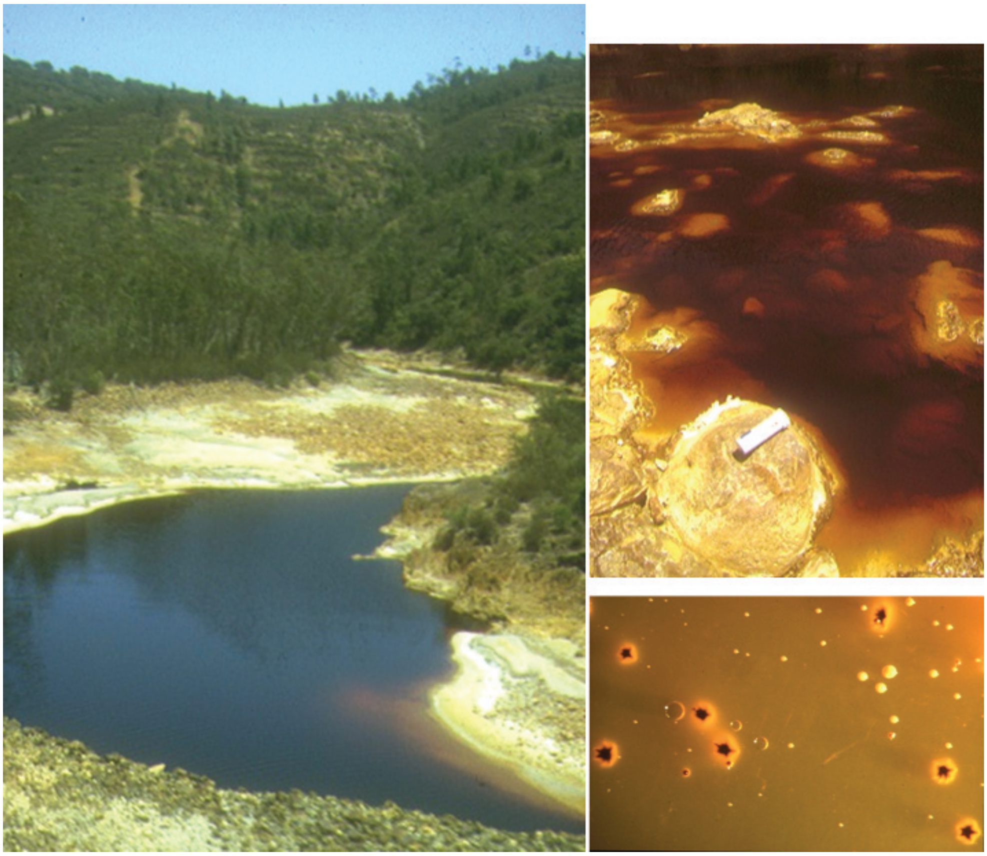

Figure 3 The Rio Tinto, an iron-rich extremely acidic river that flows though southwest Spain (left); the deep red-colored water of the Rio Tinto, due to the presence of elevated concentrations of soluble ferric iron (top right); colonies of rust-colored iron-oxidizing bacteria (*Acidithiobacillus ferrooxidans*) and heterotrophic acidophiles (*Acidiphilium* spp.) isolated from the Rio Tinto (bottom right).

Apart from their characteristic low pH and elevated metal contents, the chemistries of mine waters are highly variable, as described in the section titled 'Acid mine streams and lakes'. However, concentrations of inorganic nitrogen (generally exclusively ammonium, except where there is input of nitrate from rock blasting at working mines), phosphate, and dissolved organic carbon all tend to be relatively small.

Biodiversity of Extreme Acidophiles

Extremely acidophilic organisms are exclusively microbial and include both prokaryotes and eukaryotes. The axiom that as an environmental parameter (in this case, acidity) becomes more extreme biodiversity declines holds true for both groups. Although some angiosperms have been observed to grow in highly acidic lakes, their root systems grow in sediments in which the pH is usually much higher than the water body itself. Many eukaryotic microorganisms that have been observed in extremely low-pH environments are acid-tolerant rather than truly acidophilic, and may grow equally well, or better, in circum-neutral pH environments.

Primary Producers in Acidic Environments

The first extremely acidophilic microorganism to be isolated and characterized was the sulfur-oxidizing bacterium, *Acidithiobacillus* (*At.*) *thiooxidans* (then referred to as *Thiobacillus thiooxidans*) by Waksman and Joffe in 1921. Some years later, another sulfur-oxidizing bacterium was isolated from water draining a coal mine that had the unique trait (at the time) of also being able to oxidize ferrous iron to ferric. (*Acidi*)*thiobacillus ferrooxidans* has subsequently become the most well studied of all acidophilic microorganisms. Both of these early isolates are autotrophic chemolithotrophs, that is, they use inorganic electron donors and fixed carbon dioxide. Such a metabolic lifestyle is highly appropriate in extremely acidic environments, which, as noted previously, tend to contain elevated concentrations of potential inorganic energy sources (ferrous iron and reduced sulfur) but often low concentrations of dissolved organic carbon. While more recent studies have led to the isolation of a number of other genera and species of chemolitho-autotrophic acidophiles, a large number of highly biodiverse microorganisms that have very different metabolic lifestyles (e.g., phototrophic microalgae, heterotrophic bacteria and yeasts, and phagotrophic protozoa) have also been shown to be obligate acidophiles (see 'Acidophilic eukaryotic microorganisms').

Primary production (net assimilation of carbon) in extremely acidic environments is carried out by two main groups of microorganisms, the relative importance of which varies from site to site. Chemolitho-autotrophic acidophiles are CO_2 fixers that use either ferrous iron or reduced sulfur (or, in some cases, both) as energy sources. Sulfide (e.g., in minerals), elemental sulfur, and RISCs are far more energetic substrates than ferrous iron (**Table 1**) though interestingly at least one bacterium (*At. ferrooxidans*) that can use both ferrous iron and sulfur as substrates appears to opt for the former when both are available. Some acidophiles have also been shown to use hydrogen as an electron donor. The significance of this is unknown, though hydrogen may form in these environments from reactions between protons and various minerals in contact with acidic liquors. Other chemolitho-autotrophic metabolisms (e.g., nitrification) have not been observed in extremely acidic environments. Photoautotrophy (the use of solar energy to fuel carbon dioxide fixation) may be the dominant mechanism of primary production in acidic ecosystems, as in most others, though in underground sites (acidic caves and mine caverns) primary production is exclusively mediated by chemolithotrophs. All acidophilic phototrophic microorganisms that have been identified are eukaryotic microalgae. No truly acidophilic phototrophic bacteria (aerobic cyanobacteria or anaerobic purple/green S-bacteria) have been described, though clones of anaerobic photosynthetic green sulfur and purple nonsulfur bacteria have been obtained from an acidic geothermal site in New Zealand (see 'Geothermal areas').

Many acidophilic microorganisms that fix carbon dioxide are obligate autotrophs. Some, however, can switch to assimilating organic carbon if and when this

Table 1 Comparison of free energy changes associated with the oxidation of inorganic substrates used by chemolithotrophic acidophiles

Reaction	*Free energy change ΔG^o (kJ mole substrate^{-1})*
Ferrous iron oxidation	
$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O$	–30 (at pH 2.0)
Hydrogen oxidation	
$H_2 + 0.5O_2 \rightarrow H_2O$	–237
Elemental sulfur oxidation	
$S^0 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$	–507
Hydrogen sulfide oxidation	
$H_2S + 2O_2 \rightarrow H_2SO_4$	–714
RISC oxidation	
(1) $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$	–739
(2) $S_4O_6^{2-} + 3.5O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+$	–1225

Source: Data from Kelly DP (1978) Bioenergetics of chemolithotrophic bacteria. In: Bull AT and Meadows PM (eds.) *Companion to Microbiology*, pp. 363–386. London: Longman, and Kelly DP (1999) Thermodynamic aspects of energy conservation by chemolithotrophic bacteria in relation to the sulfur oxidation pathways. *Archives of Microbiology* 171: 219–229.

becomes available. The metabolic logic for this is obvious, as CO_2 fixation is a highly energy-consuming process (e.g., *At. ferrooxidans* has been estimated to utilize most of the energy it obtains by oxidizing iron on this single process), and using prefixed carbon, assuming that it is readily incorporated and metabolized, avoids this expenditure of energy. Various terms have been used to describe such microorganisms, which include some eukaryotic algae as well as some prokaryotic acidophiles, though the most appropriate (and least ambiguous) is to refer to them as facultative autotrophs. Whether such acidophiles are net contributors to total primary production depends not only on the presence of metabolizable organic carbon, but also (in the case of phototrophs such as *Cyanidium caldarium*) on the availability of solar energy.

Heterotrophic Acidophiles

Unusually for microbial ecology, the first obligately heterotrophic acidophilic bacteria (*Acidiphilium* spp.) were isolated some 70 years after the first chemolithotrophic acidophile, though a heterotrophic acidophilic archaeon (*Thermoplasma* (*Tp.*) *acidophilum*) was actually described a decade before the first *Acidiphilium* sp. (*Acidiphilium cryptum*). There are now a large number of characterized species of acidophilic bacteria and archaea that are known to use organic compounds as sources of both carbon and energy. Some of these are able to supplement their energy budgets by oxidizing inorganic substrates (ferrous iron or reduced sulfur) when these are also available. In the case of truly mixotrophic acidophiles, such as the iron-oxidizing heterotroph *Ferrimicrobium* (*Fm.*) *acidiphilum*, the inorganic substrate can serve as the sole source of energy and the organic moiety only to meet the carbon requirements of the bacterium.

Bacteria, in particular, are renowned as a collective group of microorganisms for their abilities to degrade a multitude of small and large molecular weight organic compounds, including many synthetic materials. Acidophilic prokaryotes, on the other hand, appear to use a far more restricted range of monomeric organic substrates and few polymeric materials. Simple sugars and alcohols are utilized by many heterotrophic acidophiles, but aliphatic acids (such as acetic acid) tend to be lethal to acidophiles when present in only micromolar concentrations. The reason for this relates to the fact that many small molecular weight organic acids exist as undissociated, lipophilic molecules in low-pH liquors. These can freely permeate microbial membranes and accumulate in the circum-neutral pH cell interiors where they dissociate and cause intracellular acidification of the cytoplasm. Di- and tricarboxylic organic acids, such as citric acid, are not so toxic and are actually used as substrates by many heterotrophic acidophiles. Some organic acids, most notably glutamic acid, also serve as appropriate substrates for many acidophiles, though others (e.g., glycine) do not. Complex, nitrogen-rich organic substrates, such as yeast extract and tryptone, are also suitable substrates for isolating and cultivating many heterotrophic acidophiles and supplementing defined organic growth media with, for example, yeast extract often promotes growth of heterotrophic acidophilic bacteria and archaea. One of the few known examples of an acidophile being able to grow on an organic polymer is the archaeon *Acidilobus aceticus*, which grows anaerobically on starch, forming acetate as the main metabolic product.

Aerobic and Anaerobic Acidophiles

The majority of known acidophilic prokaryotes have been classed as obligate aerobes. More detailed examination has revealed that, in a number of cases, they can also grow in the absence of oxygen and are therefore facultative anaerobes. Of the various options that microorganisms use for living in the absence of oxygen, by far the most widespread among acidophiles appears to be ferric iron respiration. This is understandable since iron, as both ferrous and ferric, is usually abundant in extremely acidic environments, particularly those originating from the oxidative dissolution of sulfide minerals. There is also a thermodynamic advantage to be gained from using ferric iron in that the redox potential (E_h value) of the ferrous/ferric couple at low pH is about +770 mV, a value which is not much below that of the oxygen/water couple (+840 mV) and considerably more positive than alternative inorganic electron acceptors such nitrate and sulfate. Most Bacteria (and the Euryarchaeote *Ferroplasma* (*Fp.*) *acidiphilum*) that can oxidize ferrous iron in the presence of molecular oxygen can also reduce it when oxygen is absent. Notable exceptions are species of *Leptospirillum*, though this is explained by the fact that these highly specialized bacteria have not been found to use an electron donor other than ferrous iron. Other acidophilic bacteria that also reduce ferric iron to ferrous are obligate heterotrophs, one of which *Fm. acidiphilum* is an iron oxidizer, while others (all species of *Acidiphilium*, as well as many *Acidocella* and *Acidobacterium* spp.) are not. This trait is not universal among acidophilic heterotrophic bacteria, however, as illustrated by the fact that *Acidisphaera rubrifaciens* and closely related isolates do not appear to reduce ferric iron. The earlier claim that the sulfur-oxidizing bacterium *At. thiooxidans* can reduce ferric iron was later challenged as probably being an artifact resulting from chemical reduction by RISCs that are produced during sulfur metabolism. The thermotolerant sulfur oxidizer *Acidithiobacillus caldus* also does not appear to reduce ferric iron.

Sulfur respiration (the use of elemental sulfur as electron acceptor) is not uncommon among acidophilic archaea: *Acidianus, Stygiolobus, Sulfurisphaera* (all thermoacidophilic crenarchaeotes), and *Thermoplasma* (a moderately thermoacidophilic euryarchaeote) can all grow anaerobically by reducing sulfur to hydrogen sulfide. *Acidianus* spp. and *Sulfurisphaera ohwakuensis* are both facultative anaerobes that couple the oxidation of hydrogen to the reduction of sulfur in anoxic environments, while *Stygiolobus azoricus* is an obligately anaerobic thermoacidophile that can do the same. In contrast, both classified species of *Thermoplasma* (*Tp. acidophilum* and *Tp. volcanium*) are facultative anaerobes that couple the oxidation of organic carbon to the reduction of elemental sulfur. No extremely acidophilic sulfur- or sulfate-reducing bacteria (SRB), or sulfate-reducing archaea, have yet been isolated and characterized, though there is evidence that sulfidogens are both present and active in some anaerobic acidic environments. A *Desulfosporosinus*-like isolate (M1), isolated from a geothermal site on Montserrat, West Indies, has been demonstrated to grow in a mixed culture at pH 3.2 and above, but is probably acid-tolerant rather than a true acidophile. Many other apparently acid-tolerant sulfidogenic isolates and putative clones detected in acidic mine waters have also been found to be Gram-positive bacteria.

Clones of methanogenic archaea have also been identified in gene libraries constructed from DNA extracted from some extremely acidic environments, but no extremely acidophilic methanogens are known. Likewise, no acetogenic acidophiles have been isolated, though this may be explained on the biotoxicity of acetic acid in low-pH liquors, as discussed previously. The general toxicity of aliphatic acids may also help account for the apparent absence of fermentative metabolism among extreme acidophiles, with the exception of the thermophilic archaeon *A. aceticus*, which can grow by fermenting starch to acetic acid. The scarceness of nitrate in most acidic environments, apart from those in the vicinity of rock blasting, and the fact that acidophiles are more sensitive to nitrate and nitrite than most other bacteria are probably why nitrate respiration is apparently absent in these microorganisms.

Temperature and pH Characteristics of Acidophilic Microorganisms

One of the most widely used methods to categorize acidophilic prokaryotes is on the basis of their temperature characteristics, that is, their optimum temperatures for growth and the range of temperatures within which they are active (**Table 2**). Three groups of acidophiles have often been recognized in this way: (1) mesophiles, with temperature optima of 20–40 °C; (2) moderate

Table 2 Categorization of validated species and genera of extremely acidophilic prokaryotic microorganisms, based on growth temperature optima

	Carbon assimilation	*Fe^{2+} oxidation*	*Fe^{3+} reduction*	*S^0 oxidation*	*S^0 reduction*
(a) Mesophiles (temperature optima 20–40 °C)					
At. ferrooxidans	OA	+	+	+	+
L. ferrooxidans	OA	+	−	−	−
Fm. acidiphilum	OH	+	+	−	−
At. thiooxidans	OA	−	−	+	−
Thiomonas spp.	FA	+	−	+	−
Acidiphilium spp.	OH	−	+	+	−
A. acidophilum	FA	−	+	+	−
Acidocella spp.	OH	−	+	−	−
Acidobacterium spp.	OH	−	+	−	−
Fp. acidiphilum	OH	+	+	−	−
(b) Moderate thermophiles (temperature optima 40–60 °C)					
L. ferriphilum	OA	+	−	−	−
Sulfobacillus spp.	FA	+	+	+	−
Alicyclobacillus spp.[a]	OH/FA	+/−	+/−	+/−	−
Am. ferrooxidans	FA	+	+	−	−
Fx. thermotolerans	OH	+	+	−	−
Acd. organivorans	OH	−	−	+	−
At. caldus	OA	−	−	+	−
Thermoplasma spp.	OH	−	−	−	+
Picrophilus spp.	OH	−	−	−	−
(c) Extreme thermophiles (temperature optima >60 °C)					
H. acidophilum	OA	−	−	+	−
S. acidocaldarius	OH	−	−	−	−
S. solfataricus	OH	−	−	−	−
S. metallicus	OA	+	−	+	−
S. tokodaii	OH	+	−	+	−

(*Continued*)

Table 2 (Continued)

	Carbon assimilation	*Fe^{2+} oxidation*	*Fe^{3+} reduction*	*S^0 oxidation*	*S^0 reduction*
Metallosphaera spp.	FA	−	−	+	−
Sulfurococcus spp.	FA	−	−	+	−
A. infernus	OA	−	−	+	+
Ac. ambivalens	OA	−	−	+	+
Ac. brierleyi	FA	+	−	+	+
Sg. azoricus	OA	−	−	−	+
Ss. ohwakuensis	FA	−	−	−	+

[a] *Alicyclobacillus* spp. include species that are facultatively autotrophic and obligately heterotrophic, and vary in terms of their dissimilatory transformations of iron and sulfur.

Note: OA, obligate autotroph; FA, facultative autotroph; OH, obligate heterotroph.

Genera abbreviations: *At., Acidithiobacillus; L., Leptospirillum; Fm., Ferrimicrobium; A., Acidiphilum; Sb., Sulfobacillus; Fp., Ferroplasma; Am., Acidimicrobium; Fx., Ferrithrix; Acd., Acidicaldus; H., Hydrogenobaculum; S., Sulfolobus; Ac., Acidianus; Sg., Stygiolobus; Ss., Sulfurisphaera.*

thermophiles, with temperature optima of 40–60 °C; and (3) extreme thermophiles, with temperature optima of 60–80 °C. While some acidophiles (strains of *At. ferrooxidans* and *Acidiphilium*) have been demonstrated to be active at very low (<5 °C) temperatures, all of these have temperature optima well above 20 °C, and are therefore psychrotolerant rather than psychrophilic microorganisms. At the other end of the temperature spectrum, the most thermophilic extreme acidophile known is the facultatively anaerobic sulfur-metabolizing archaeon *Acidianus infernus*, which has a growth temperature optimum of 90 °C and a maximum of about 96 °C. However, relatively few hyperthermophilic acidophiles are known and the fact that the maximum temperature for growth of an acidophile is about 25 °C lower than that of the most thermophilic life-forms known (neutrophilic *Pyrolobus*-like archaea) is possibly a reflection of the difficulty that living organisms have when challenged by the dual stresses of extreme temperature and acidity. In addition, the pH of high-temperature (>100 °C) abyssal environments around submarine vents is maintained at close to neutral by the strong buffering capacity of seawater, precluding extensive colonization by acidophiles.

As with neutrophilic prokaryotes, extremely thermophilic acidophiles are mostly Archaea while mesophiles are predominantly Bacteria. The majority of moderate thermoacidophiles are also Bacteria, and mostly Gram-positives, while most known Gram-negative acidophilic bacteria grow best at below 40 °C. There are exceptions to this general trend. Indeed, the most thermophilic acidophilic bacteria known – the sulfur-oxidizing autotroph *Hydrogenobaculum acidophilum*, which grows at up to 70 °C, and the heterotroph *Acidicaldus organivorans*, which grows at up to 65 °C – are both Gram-negative.

The ability to tolerate elevated concentrations of protons (strictly speaking, hydronium ions; H_3O^+) is obviously what defines an acidophile. While there is no official cutoff pH value that delineates whether an organism is or is not an acidophile, the generally accepted view is that, as a group, these can be divided into extreme acidophiles that have pH optima for growth at pH < 3, and moderate acidophiles that have pH optima of between 3 and 5. As can be anticipated, the most extremely acidic environments have less potential biodiversity than those that are moderately acidic. The number of prokaryotes that are known to grow at pH < 1 is relatively small and includes some Gram-positive bacteria (e.g., *Sulfobacillus* spp.), Gram-negative bacteria (e.g., *Leptospirillum* spp. and *At. thiooxidans*), and Archaea (e.g., *Ferroplasma* spp.) that oxidize iron and/or sulfur. The most acidophilic of all currently known life-forms is, however, a heterotrophic archaeon, *Picrophilus*. Two species are known, *Picrophilus oshimae* and *Picrophilus torridus*, both of which have optima pH for growth of ~0.7, and grow in synthetic media poised at pH ~ 0. These 'hyperacidophiles' are also thermophilic, with optimum temperatures for growth at ~60 °C.

Physiological Versatility in Acidophilic Prokaryotes: Specialized and Generalist Microorganisms

Acidophiles as a group are highly versatile and are able to utilize a wide variety of energy sources (solar and inorganic and organic chemicals), grow in the presence or complete absence of oxygen, and at temperatures of between 4 and 96 °C. However, individual species display very different degrees of metabolic versatility. On the one end of this spectrum are members of the genus *Leptospirillum*. Three species are known: *Leptospirillum ferrooxidans, Leptospirillum ferriphilum*, and *Leptospirillum ferrodiazotrophum*. All grow as highly motile curved rods and spirilli, and species and strains vary in temperature and pH characteristics. All three species, however, appear to use only one energy source – ferrous iron. Because of the high redox potential of the ferrous/ferric couple (see 'Aerobic and anaerobic acidophiles'), these Bacteria, by necessity, have to use molecular oxygen as an electron acceptor, restricting them to being active only in aerobic environments. All three species fix carbon dioxide (but not organic carbon) and two of the three (*L. ferrooxidans* and *L. ferrodiazotrophum*) are also able to fix molecular nitrogen. *Leptospirillum* spp. are, therefore, highly specialized acidophiles. Their metabolic limitations appear, however, to be compensated by their abilities to outcompete other iron-oxidizing bacteria in many natural and anthropogenic environments, such as stirred tank bioreactors used to bioleach or biooxidize sulfide ores. This is achieved, at least in part, by their greater affinities for ferrous iron and greater tolerance of ferric iron than most other iron oxidizers.

At. ferrooxidans is, in contrast, a more generalist bacterium. Initially it was described as an obligate aerobe that obtains energy by oxidizing ferrous iron, elemental sulfur, sulfide, and RISCs, and fixes CO_2 as its sole source of carbon. The first hint of a more extensive metabolic potential was in a report by Thomas Brock and John Gustafson in 1976 who showed that the bacterium could couple the oxidation of elemental sulfur to the reduction of ferric iron, though it was not confirmed at the time whether this could support growth of the acidophile in the absence of oxygen, though the free energy of the reaction ($\Delta G = -314\,\mathrm{kJ\,mol^{-1}}$; eqn [6]) suggested that this might be the case.

$$S + 6Fe^{3+} + 4H_2O \rightarrow HSO_4^{-} + 6Fe^{2+} + 7H^{+} \qquad (6)$$

Later, Jack Pronk and colleagues at Delft University showed conclusively that *At. ferrooxidans* is, indeed, a facultative anaerobe and can grow anaerobically by ferric iron respiration using not only sulfur as electron donor, but also formic acid (which can also be used as sole energy source under aerobic conditions). The finding that this acidophile can use formic acid, although somewhat unexpected, does not imply that it is capable of heterotrophic as well as autotrophic growth, as C_1 compounds, such as formate and methanol, are also used by other autotrophic prokaryotes. About the same time, it was discovered that some strains of *At. ferrooxidans* (including the type strain) can use hydrogen as an energy source, but that bacteria cultivated on hydrogen are less acidophilic than when grown on sulfide ores. It was shown later that hydrogen oxidation could also be coupled to ferric iron reduction by some *At. ferrooxidans* isolates.

The most generalist of all acidophiles are, however, *Sulfobacillus* spp. These Gram-positive bacteria can grow as chemolithotrophs, heterotrophs, or mixotrophs in aerobic or anaerobic environments. Although there are no reports of *Sulfobacillus* spp. using hydrogen, they can (unlike *At. ferrooxidans*) use a variety of organic compounds (such as glucose and glycerol) as carbon and energy sources, though their capacities for heterotrophic growth are more limited than *Alicyclobacillus* spp. (related acidophilic Firmicutes, some of which can also oxidize ferrous iron and sulfur).

Acidophilic Eukaryotic Microorganisms

Extremely acidophilic organisms are exclusively microbial. While some angiosperms, such as *Juncus bulbosus* and *Eriophorum angustifolium*, can grow in highly acidic (pH < 3) ponds and lakes, their root systems grow in sediments where the pH is usually significantly higher than the water body itself. Many eukaryotic microorganisms that may be found in extremely low pH environments are acid-tolerant rather than truly acidophilic and may grow equally well, or better, in higher pH waters.

All known phototrophic acidophiles are eukaryotic, and both mesophilic and moderately thermophilic species are known. Some photosynthetic acidophiles are also capable of heterotrophic growth in the absence of light, provided that a suitable carbon source is available. Microalgae that can live in highly acidic environments include genera of Chlorophyta, such as *Chlamydomonas acidophila* and *Dunaliella acidophila*; Chrysophyta, such as *Ochromonas* sp.; and Euglenophyta, such as *Euglena mutabilis* (**Figure 4**). Some diatoms, including several *Eunotia* spp., have also been found to colonize extremely acidic waters. A filamentous alga, identified from its morphology as *Zygnema* and confirmed from biomolecular analysis to be *Zygnema circumcarinatum*, has been found in abundance on surface streamer growths in an extremely acidic (pH ~ 2.7) metal-rich stream draining a mine adit in southwest Spain. Four species of thermoacidophilic Rhodophyta have been described. Of these, *Galderia* spp. (*Galderia sulfuraria* and *Galderia maxima*) can grow as heterotrophs, while *Cyanidioschyzon merolae* and the original strain of *C. caldarium* are strict autotrophs. One *C. caldarium*-like isolate has been reported to grow in synthetic media poised as low as pH 0.2. *Chlorella*-like microalgae have also been detected in acidic geothermal waters.

Many species of yeasts and fungi can tolerate moderate or even extreme acidity. Truly acidophilic fungi are, however, less common, though these include some remarkable species, such as *Acontium velatum* and *Scytalidium acidophilum*, both of which are copper-tolerant mitosporic fungi that can grow at pH values of below 0.5. Among the most commonly encountered yeasts in metal-rich acidic waters are *Rhodotorula* spp., while some *Cryptococcus* spp. and *Trichosporon dulcitum* are also acidophilic yeasts. Novel acidophilic fungal isolates (proposed name *Acomyces richmondensi*) have been isolated from warm (30–50 °C), extremely acidic (pH 0.8–1.38), and iron/zinc/copper/arsenic-contaminated waters within the Richmond mine at Iron Mountain, California.

Microscopic animal life-forms may also be found in acidic environments. The most biodiverse of these appear to be protozoa (**Figure 4**). Phagotrophic flagellates

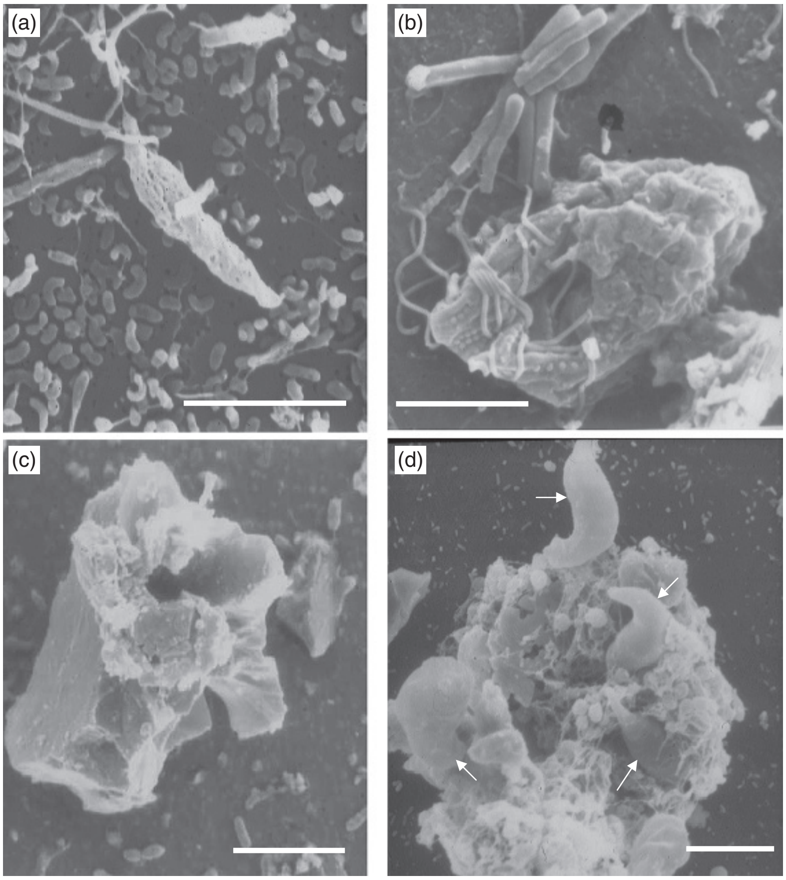

Figure 4 Scanning electron micrographs of eukaryotic acidophiles: (a) a *Eutreptia*-like flagellate protozoan, grazing on *Leptospirillum ferrooxidans*; (b) a *Cinetochilum*-like ciliate protozoan, grazing on *Acidithiobacillus ferrooxidans*; (c) a *Vahlkampfia*-like amoeboid protozoan; (d) a bundle of *Euglena mutabilis* (an acidophilic microalga) with individual cells arrowed. The scale bar represents 5 μm in micrographs (a)–(c), and 10 μm in micrograph (d).

(*Eutreptia*), ciliates (*Urotricha, Vorticella, Oxyticha*, and *Cinetochilum*), and amoeba (*Vahlkampfia*) have all been encountered in acidic mine waters, and some have also been grown in acidic media in the laboratory. Multicellular animal life-forms are relatively uncommon, though rotifers (such as *Cephalodella hoodi* and *Cephalodella gibba*) have occasionally been identified in acidic mine waters. The two most acidophilic species of known rotifers appear to be *Elosa woralii* and *Brachionus sericus*, though the latter can also grow at neutral pH *in vitro*. The pioneering crustacean *Chydorus sphaericus* has also been observed in the pelagic community of acid mine lakes in Germany, though it is acid-tolerant rather than acidophilic, with a pH range of 3.2–10.6.

Interactions Between Acidophilic Microorganisms

The study of microbial ecology involves not only understanding the impact of the environment on microorganisms (and vice versa) but also examining how microorganisms interact with each other. Along with increasing awareness of the biodiversity and complexity of life in extremely acidic environments have come fresh insights into the wide range of microbial interactions that occur within them. In some cases, such as grazing by phagotrophic protozoa on acidophilic bacteria, the interaction may be readily observed, though more often it is more clandestine.

Mutualistic Interactions

Mutualistic interactions are where both partners derive some benefit from their association. One way in which this occurs in extremely acidic environments is via redox transformations and transfer of iron and/or sulfur between prokaryotes. As noted in the section titled ‘Biodiversity of extreme acidophiles’, ferrous iron is an energy source that is widely used by acidophilic Bacteria and some acidophilic Archaea, while ferric iron can act as a highly effective alternative electron acceptor to oxygen in low pH environments. Juxtaposition of aerobic and microaerobic/anaerobic environments can lead to rapid cycling of iron between the two zones. This is aided by the fact that, in contrast to most environments, ferric iron is soluble at pH < 2.5 and is more readily utilized as an electron sink as soluble Fe^{3+} than when present in its various amorphous and crystalline forms. The importance of iron cycling has been illustrated in major acidic environments such as the Rio Tinto, and also demonstrated *in vitro*. Obviously, an extraneous energy source is required for iron cycling to perpetuate. In acidic environments, this may be organic carbon, originating as exudates and lysates from primary producers (phototrophs and chemolithotrophs) that act as electron donors for iron-reducing acidophiles. Cycling of iron may involve more than one species (e.g., the iron-oxidizer *At. ferrooxidans* and the iron-reducer *Acidiphilium*) or a single species (e.g., of *Sulfobacillus*). The situation with sulfur transformations is less clear, due in part to the relative paucity in the knowledge of bacterial sulfate/sulfur reduction in low-temperature acidic environments, and the far greater insolubility of some reduced sulfur compounds (metal sulfides and elemental sulfur) than ferric iron at extremely low pH, which limits their free diffusion. Sulfate produced by aerobic sulfur-oxidizing acidophiles (such as *Acidithiobacillus* and *Thiomonas* spp.) can diffuse into underlying sediments and act as a terminal electron acceptor for any acidophilic/acid-tolerant SRB present. These generate sulfide, which, at low pH, is present almost exclusively as gaseous H_2S. The presence in the sediments of soluble metals, such as copper, that form very insoluble sulfides, results in the rapid removal of H_2S, even at very low pH. However, if, as is often the case, the dominant soluble chalcophilic metal present is (ferrous) iron, the lower solubility of the sulfide mineral (FeS) means that it does not form until the pH has risen to $\sim$5. If the sediment pH is <5, at least some of the H_2S can, at least in theory, diffuse into the overlying water and act as an energy source for sulfur-oxidizing acidophiles.

Other examples of mutualistic interaction between acidophilic bacteria involve the metabolism of organic compounds. Autotrophic iron- and sulfur-oxidizing acidophiles fix carbon dioxide to incorporate into cell biomass. Some of this, mostly small molecular weight material, is lost from actively metabolizing cells and has been shown to accumulate in axenic cultures of these prokaryotes grown in the laboratory. Additional organic carbon originates from dead and dying cells as cell lysates. In the environment, much of this organic carbon is metabolized by heterotrophic acidophiles, such as *Acidiphilium* spp., which are adept scavengers. Positive feedback to the autotrophs comes from the fact that many of these are sensitive to small molecular weight organic compounds in general, and aliphatic acids in particular, and the catabolism of these materials by heterotrophic acidophiles therefore reduces or eliminates this potential toxicity hazard. Iron-oxidizing acidophiles vary in their sensitivities to organic materials; *L. ferrooxidans* is, for example, much more sensitive than *At. ferrooxidans*. This is reflected in the far greater mortality rate of the former in spent (substrate-depleted) media, and is the reason why mixed cultures of *L. ferrooxidans* and *Acidiphilium* spp., grown on ferrous iron or pyrite, tend to be far more stable than pure cultures of the iron oxidizer. In practical terms, the inclusion of obligately or facultatively heterotrophic acidophiles in mineral-leaching consortia has been shown to improve metal recovery, and commercial-scale stirred tanks used to bioprocess sulfide ores have invariably been found to include organic carbon-degrading acidophiles as well as those that fix CO_2. The same rationale of using heterotrophic acidophiles to remove potentially toxic organic materials has been used to develop solid media for isolating and cultivating iron- and sulfur-oxidizing acidophiles from environmental and industrial samples.

Synergistic Interactions

Microbial interactions that result in the complimentary activities of both (or all) participants being more efficient in, for example, degrading a substrate, than the individual species working alone, are referred to as synergistic. One such example involves the oxidative dissolution of pyrite by mixed cultures of *L. ferrooxidans* and *At. thiooxidans*. *L. ferrooxidans* is an iron oxidizer that is unable to oxidize sulfur, while *At. thiooxidans* has the opposite abilities. Pyrite, being an acid-insoluble sulfide mineral (see 'Mine-impacted environments'), is oxidized by ferric iron produced by ferrous iron-oxidizing *L. ferrooxidans* in an acid-consuming reaction. The RISCs produced as a result of ferric iron attack on pyrite are oxidized to sulfuric acid by *At. thiooxidans*, thereby generating the extremely low pH conditions under which *L. ferrooxidans* thrives and mineral dissolution is accelerated. Pure cultures of *L. ferrooxidans* are, however, also able to accelerate the oxidative dissolution of pyrite, in contrast to the heterotrophic iron oxidizer *Fm. acidiphilum*, which requires a source of organic material, such as yeast extract, as carbon source. When *Fm. acidiphilum* and *At. thiooxidans* are grown in mixed culture, the interaction involves carbon transfer as well as the modulation of acidity. *Fm. acidiphilum* can obtain sufficient carbon to grow from autotrophic partners, such as *At. thiooxidans*. This facilitates ferric iron generation, producing RISCs from the degrading pyrite that serves as the energy source for *At. thiooxidans* and continued fixation of CO_2 and release of organic carbon (**Figure 5**).

Syntrophic Interactions

In syntrophic relationships, the degradation of a substrate by one species is made thermodynamically possible through the removal of an end product by another species. Neutrophilic SRB have frequently been reported as one of the partners in a syntrophic association. Hydrogen is a common end product of fermentative metabolism and the oxidation of this gas, coupled to sulfate reduction, may

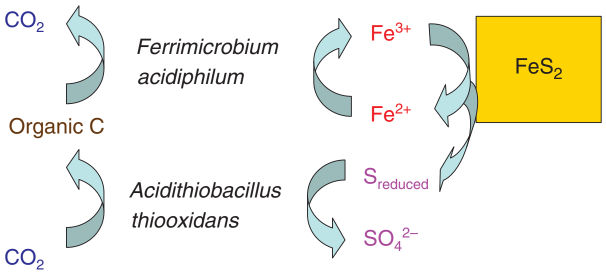

Figure 5 Dissolution of pyrite by a mixed culture of *Ferrimicrobium* (*Fm.*) *acidiphilum* and *Acidithiobacillus* (*At.*) *thiooxidans*. Though *Fm. acidiphilum* can oxidize ferrous iron to ferric iron (which is the chemical that directly attacks the pyrite mineral), it requires organic carbon to grow. *At. thiooxidans* can fix CO_2 and releases some of this as organic carbon, but it cannot access its energy source (reduced sulfur) directly from pyrite. Therefore, neither bacterium can grow in organic carbon-free pure cultures, using pyrite as an energy source, but together they form a successful synergistic consortium, via the interactions shown. Modified from Bacelar-Nicolau P and Johnson DB (1999) Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. *Applied and Environmental Microbiology* 65: 585–590, with permission from the publisher.

result in a change in the overall free energy (ΔG) and allow an otherwise thermodynamically unfeasible reaction to proceed. A syntrophic association involving an acid-tolerant *Desulfosporosinus*-like SRB and an acetic acid-degrading *Acidocella* sp. has been proposed to account for sulfidogenesis in moderately acidic (pH 3.2 and above) media. This mixed culture grows anaerobically using glycerol as sole carbon and energy source, a substrate that the SRB can oxidize but the *Acidocella* cannot. In pure cultures of the *Desulfosporosinus*, acetic acid accumulates in equimolar proportion to the amount of glycerol oxidized, but in the presence of *Acidocella* the appearance of acetic acid is transient and more sulfide is produced. It was postulated that acetic acid is degraded to hydrogen and carbon dioxide, a reaction that is only feasible in thermodynamic terms if at least one of these products is rapidly removed. This role was fulfilled by the *Desulfosporosinus* sp., which was shown to use hydrogen as well as glycerol as an electron donor. The energetic bonus for the SRB (hydrogen), which was only available in the mixed culture, resulted in it generating more hydrogen sulfide than when it was grown in pure culture. Additional support for the hypothesis came from the observation that the mixed culture, but not axenic cultures of the *Desulfosporosinus* isolate, could use acetic acid to fuel sulfidogenesis.

Predation

The fact that some acidophilic Bacteria are predated by protozoa and rotifers has been known for many years. One of the first indicators that some protozoa could grow in highly acidic waters was a report in 1941, where it was noted that the flagellate *Polytomella caeca* could grow over a wide pH range (from 1.7 to 9.2). Early studies of acid mine drainage (AMD) frequently reported the presence of flagellates, ciliates, and amoeba, and the first laboratory study was by Henry Ehrlich at the Rensselaer Polytechnic Institute (in 1963) who found that a *Eutreptia*-like flagellate could grow in enrichment cultures prepared using mine water as an inoculum. A detailed study of another *Eutreptia*-like flagellate was described some 30 years later. This protozoan was found to be obligately acidophilic, with a pH range of 1.8–4.5 for growth. Although it was highly sensitive to some heavy metals (e.g., copper, silver, and molybdenum), it could tolerate very high concentrations of both ferrous and ferric iron. The flagellate was found to graze a wide range of acidophilic bacteria, including *At. ferrooxidans, L. ferrooxidans*, and *A. cryptum*. It was noted that the highly motile iron oxidizer *L. ferrooxidans* was less effectively grazed than the less motile acidophile *At. ferrooxidans*, leading to mixed cultures of the two bacteria being dominated by *L. ferrooxidans* when the protozoan was present. Filamentous growth by some acidophiles also appeared to give them some protection from predation by the flagellate. Detailed examination of mixed cultures of acidophilic bacteria and five different acidophilic protozoan isolates (three flagellates, and *Cinetochilum*-like ciliate, and a *Vahlkampfia*-like amoeba) showed that, in each case, population dynamics followed classic predator–prey population dynamics (e.g., **Figure 6**).

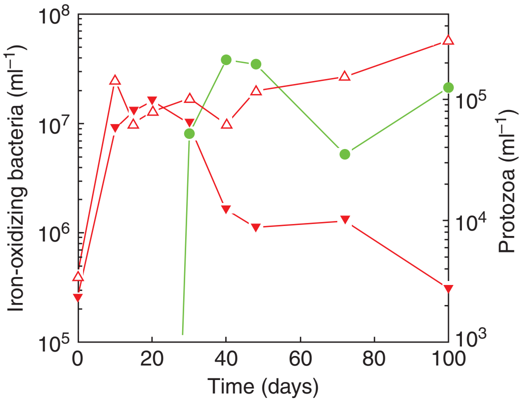

Figure 6 Grazing of acidophilic iron-oxidizing bacteria (a mixed culture of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*) by a *Cinetochilum*-like acidophilic ciliate protozoan, showing a classic predator–prey relationship. Numbers of iron-oxidizing bacteria are shown in red: solid symbols show data from a ciliate-containing culture, while hollow symbols show data from a corresponding culture where protozoa were absent. Protozoan numbers are shown in green. Modified from Johnson DB and Rang L (1993) Effects of acidophilic protozoa on populations of metal-mobilizing bacteria during the leaching of pyritic coal. *Journal of General Microbiology* 139: 1417–1423, with permission from the publisher.

Early attempts to cultivate the acidophilic protozoa in media containing pyrite failed, even though large populations of iron-oxidizing and other bacteria were present. This was later shown to be due to the fine size (<61 μm) of the pyrite grains used. When coarser-grain (61–200 μm) pyrite particles were used, all five protozoa were able to grow effectively, suggesting that the phagotrophic protozoa were unable to differentiate between pyrite and bacteria, and that inadvertent ingestion of bacteria-sized pyrite grains resulted in death of the protozoa. Interestingly, dramatic reductions of numbers of iron-oxidizing bacteria due to protozoan grazing did not necessarily result in decreased rates of pyrite dissolution, possibly because of the overriding influence of mineral-oxidizing bacteria attached to the pyrite, which were not grazed.

Evidence for predation of acidophilic bacteria by other microorganisms has come mostly from microscopic observations. Rotifers, for example, have been seen to feed on acid streamer microbial communities (see 'Acidophilic eukaryotic microorganisms') using their wheel-like cilia to draw a vortex of bacterial cells into their mouths.

Competitive Interactions

As might be anticipated, competition between acidophiles for electron donors and acceptors, inorganic nutrients, and so on, is as important in acidic as in all other environments. One of the most detailed studies of this kind, given the importance of iron-oxidizing bacteria in commercial mineral processing and the genesis of AMD, has been the competition between *At. ferrooxidans* and *Leptospirillum* spp. for their communal substrate (electron donor), ferrous iron. Early assumptions that *At. ferrooxidans* was invariably the dominant iron-oxidizing bacterium in metal-rich, acidic environments have gradually been eroded, with increasing numbers of reports describing *L. ferrooxidans* (though in some cases this is probably *L. ferriphilum*) as the more abundant species. These include mine drainage waters and some (mostly stirred tank) commercial biomining operations. In general, Bacteria classified as *At. ferrooxidans* tend to grow more rapidly than *Leptospirillum* spp., and are better able to exploit acidic environments that contain relatively large concentrations of ferrous iron. In terms often used to differentiate heterotrophic bacteria, *At. ferrooxidans* is a copiotroph while *Leptospirillum* spp. are oligotrophs. This is also the reason why using ferrous iron-rich synthetic media to enrich for iron-oxidizing acidophiles favors *At. ferrooxidans* rather than *L. ferrooxidans*. On the other hand, the greater affinity for ferrous iron and the greater tolerance of ferric iron of *L. ferrooxidans* (and probably other *Leptospirillum* spp.) facilitates their dominance in stirred tank mineral leachates, where ferric iron concentrations can be many grams per liter and, conversely, in those extremely acidic environments ($pH < 2.3$) where ferrous iron concentrations are very small. In both situations, redox potentials (which are determined by the relative concentrations, rather than actual concentrations, of ferrous and ferric iron) are commonly above 750 mV, and *Leptospirillum* spp. are known to be far more efficient iron oxidizers than *At. ferrooxidans* under such highly oxidizing conditions. Other important factors that affect competition between these iron-oxidizing autotrophs are temperature and pH. *Leptospirillum* spp. in general (and *L. ferriphilum* in particular) tend to be more thermotolerant than *At. ferrooxidans*, which partly explains their greater importance within the warm interior of the Richmond mine at Iron Mountain (see 'Acid mine streams and lakes') and in stirred tanks used to bioprocess gold and cobaltiferous ores, which generally operate at around 40 °C. On the other hand, cold-tolerant iron-oxidizing acidophiles have been invariably identified as *At. ferrooxidans*-like. *Leptospirillum* spp. also tend to be more tolerant of extreme acidity (many stains grow at pH 1) than *At. ferrooxidans*, some strains of which do not grow below pH 1.8, though others, including the type strain, can grow at pH 1.5. The higher pH optima for their growth is one of the reasons why *At. ferrooxidans* is often more important in heap leaching of mineral ores, as engineered mineral heaps are generally not so acidic as stirred tanks.

In one of the few studies to describe competition between two other iron-oxidizing acidophiles, dissolution of pyrite at 45 °C by a mixed culture of the thermotolerant facultatively autotrophic bacterium *Acidimicrobium* (*Am.*) *ferrooxidans* and a thermotolerant strain of the obligate autotroph *L. ferriphilum* was examined. Numbers of the two bacteria (estimated using fluorescent *in situ* hybridization; FISH) remained very similar until the pH of the bioreactor was lowered from 1.5 to 1.2, at which point *L. ferriphilum* emerged as the dominant bacterium. However, when the thermotolerant sulfur oxidizer *At. caldus* was also included in the microbial consortium, *Am. ferrooxidans* was more abundant than *L. ferriphilum* at pH 1.5 and 1.2. The reason for this was probably the additional amount of organic carbon available for the heterotrophically inclined *Am. ferrooxidans* originating from the CO_2-fixing *At. caldus* that used the RISCs produced by ferric iron attack of the pyrite in the bioreactor.

Microbial Ecology of Extremely Acidic Environments

Geothermal Areas

Geothermal areas occur is discreet zones in various parts of the world, as noted in the section titled "Nature and origin of extremely acidic environments". Probably the most well-studied land-based geothermal area is Yellowstone National Park, Wyoming, USA. Much of the early pioneering microbiological work in Yellowstone was carried out by Thomas Brock and coworkers in the 1970s and formed the basis of later research on thermophilic microorganisms (including thermoacidophiles). One of the major breakthroughs around the time was the isolation, by James Brierley, of the first thermophilic acidophile (a *Sulfolobus*-like archaeon, though it was not recognized as such at the time) from an acid hot spring in Yellowstone. Two of the more important solfatara fields in Yellowstone are located in and around the Norris Geyser Basin, and at Sylvan Springs, though there are numerous other smaller scale, often ephemeral sites (such as around the Gibbon River) where acidic ponds and streams may be found. Moderately thermophilic and acidophilic phototrophs (*Galderia*/*Cyanidium*-like rhodophytes) and Gram-positive bacteria (*Sulfobacillus* and *Alicyclobacillus* spp.) have frequently been isolated from these sites. A greater biodiversity was revealed in a study reported in 2003, where strains of what turned out to be novel genera of thermophilic Gram-negative bacteria (*Acidicaldus*), and moderately thermophilic Gram-positive bacteria (*Ferrithrix*) were isolated, as well as novel strains of *Firmicutes* that have not, as yet, been formally classified. Interestingly, many of the moderately thermophilic bacteria isolated from these sites (where the acidity is derived chiefly from the oxidation of sulfur) have been shown to catalyze the oxidation or reduction (and, in some cases, both) of iron. A study of acidic geothermal springs within the Norris Geyser Basin that contained a variety of electron donors that support the growth of chemolithotrophic acidophiles (hydrogen, hydrogen sulfide, arsenic(III), and ferrous iron) revealed complex and changing microbial communities that were determined, at least in part, by changing chemical gradients, which in turn effected major geochemical transformations. It was found that (1) *Hydrogenobaculum* (a hydrogen and sulfur oxidizer) and *Stygiolobus* (a hydrogen-oxidizing anaerobe) were present in the high temperature (~79 °C) source waters; (2) *Hydrogenobaculum* and *Thiomonas* (a ferrous iron, sulfur, and arsenic(III) oxidizer) were present in zones of rapid As(III) oxidation; and (3) *Metallosphaera* (a sulfur oxidizer), *Acidimicrobium* (an iron oxidizer), and *Thiomonas* were present in areas where As(V)-rich ferric iron oxides were being generated.

In a separate study based in the Ragged Hills area of Yellowstone, the effect of increased geothermal activity on soil microbial diversity across a temperature gradient of 35–65 °C was assessed. The pH of the soil samples analyzed ranged from 3.7 to 5.1. It was found that the DNA profiles of the soil bacteria (estimated using denaturing gradient gel electrophoresis; DGGE) in heated soils were less complex than those that had not undergone geothermal heating. The majority of clones obtained belonged to *Acidobacterium*, cultivated species of which are mostly moderate acidophiles, and mesophilic. It was concluded that thermophilic and thermotolerant microbial species are probably widely distributed in soils within Yellowstone, and that localized geothermal activity selects for them. The effects of natural hydrocarbon seeps (composed almost entirely of saturated, branched C_{15} to C_{30}, straight-and branched-chain alkanes) on the microflora of acidic (pH 2.8–3.8) sulfate-rich soils in the Rainbow Springs area of Yellowstone were examined in another study. Over 75% of the clones recovered in 16S rRNA gene libraries were related to known species of heterotrophic acidophiles (*Acidiphilum* and *Acidisphaera*) though clones related to *Acidithiobacillus* spp. were also recovered. An alkane-degrading alphaproteobacterium (distantly related to *Acidicaldus* (*Acd.*) *organivorans*, a Yellowstone isolate that has been shown to grow on phenol and other organic substrates) was isolated and partially characterized.

Other geothermal areas where the distribution of acidophilic microorganisms has been studied include New Zealand, the Caribbean island of Montserrat, and northern California. One site that has been studied in New Zealand was an acidic (pH 2.5) stream water on White Island that, in addition to soluble iron and sulfate, contained significant concentrations (2000–4400 $mg\,l^{-1}$) of chloride. Among the clones identified from DNA extracted directly from the acid stream were, unusually, those closely related to a green sulfur bacterium (*Chlorobium vibrioforme*), the marine, purple nonsulfur bacterium *Rhodovulum*, and the heterotrophic bacterium *Ralstonia solanacearum*, while those obtained from enrichment cultures also included a bacterium that was closely related to the Yellowstone isolate, *Acd. organivorans*. Pure cultures of *Acidiphilium* and *C. caldarium* were also obtained from the site. Results of a large-scale survey of geothermal sites (many of which were also extremely acidic) on Montserrat, carried our shortly prior to the major eruption of the Soufriere Hills volcano in 1996, showed that temperatures of pools and streams in the volcanic southern region of the island ranged from 30 to 99 °C, and pH from 1.0 to 7.4. Most of the acidophilic bacteria that were isolated were similar to known strains, though some *Sulfobacillus*-like isolates had novel traits in being able to grow as mesophiles, or at higher maximum growth temperatures (up to 65 °C) than classified species. Clone libraries constructed from DNA extracted from acidic sites with different temperatures indicated the presence of (1) *Acidiphilium*-like bacteria and *At. caldus* (33 °C site); (2) *At. caldus*, and a putative moderately thermophilic sulfate-reducing bacterium of the *Desulfurella* group (48 °C site); (3) novel *Ferroplasma*-like and *Sulfolobus*-like archaea (78 °C site); and (4) an archaeon distantly related to *A. infernus* (98 °C site). Elsewhere, a microbiological survey of high-temperature (82–93.5 °C) acidic (pH 1.2–2.2) hot springs located in the Lassen Volcanic National Park in northern California failed to detect any bacteria, though archaea distantly related to the crenarchaeotes *S. azoricus* and *Sulfolobus solfataricus* (both extremely acidophilic thermophiles), and others more closely related to the moderately acidophilic thermophile *Vulcanisaeta distributa*, were identified in clone libraries.

Acid Mine Streams and Lakes

Waters draining abandoned mines, mine spoils, and tailings deposits are often characterized by low pH and elevated concentrations of soluble metals (particularly iron) and sulfate (**Table 3**). These are generically referred to as AMD waters (or acid rock drainage in North America). Acidity in such waters derives from the presence of soluble aluminum, manganese, and iron (mineral acidity) as well as hydronium ions. Extremely acidic lakes may develop naturally in volcanic area, for example, Lake Kawah Idjen in Indonesia, which has a pH of ~0.7. Acidic mining lakes, in contrast, are relics of opencast mining, where worked-out voids have not been backfilled, and become progressively filled with rising groundwater or river water. Where the surrounding bedrocks are rich in sulfide minerals (normally chiefly pyrite and marcasite) and contain small amounts of carbonates, the oxidative dissolution of the former can lead to the formation of extremely acidic mine lakes. Acid mine lakes are particularly abundant in central Europe, in parts of Germany, Poland, and the Czech Republic. In past times (up to the end of the twentieth century) the extensive reserves of lignite in these areas were extracted by opencast mining on enormous scales, leaving a legacy of a very large number of man-made lakes of varying sizes and chemistries. In the Lusatia district of eastern Germany alone there are an estimated 200 mining lakes of >1 ha that have pH values of <3.

The microbiology of AMD streams has been the subject of a number of reviews in books and journals. Knowledge of how biodiverse these flowing waters can be has expanded considerably since *At. ferrooxidans* was first isolated from an AMD stream draining a bituminous coal mine in the United States in 1947. The most important factors in determining which microbial species are present in AMD appear to be pH, temperature, and concentrations of dissolved metals and other solutes. At the most extreme end of the AMD spectrum, the microbiology of mine waters within the Richmond mine at Iron Mountain, California (which can have negative pH values), has been studied extensively. Within this abandoned mine, pyrite is undergoing oxidative dissolution at a rate that is sufficient to maintain air temperatures of between 30 and 46 °C, and produce mine waters containing $\sim 200\,\mathrm{g\,l^{-1}}$ of dissolved metals. A novel iron-oxidizing archaeon, *Ferroplasma acidarmanus*, was found to be dominant in waters within the mine that had the lowest pH and highest ionic strengths, while *L. ferriphilum* and *L. ferrodiazotrophum* were also associated with exposed pyrite faces. *Sulfobacillus* spp. were more important in some of the warmer (~43 °C) waters. *At. ferrooxidans* was rarely found in sites that were in contact with the ore body, though it was found in greater abundance in the cooler, higher pH waters that were peripheral to the ore body. In contrast, a microbiological survey of much cooler and higher pH mine waters at an abandoned subarctic copper mine in Norway showed that an *At. ferrooxidans*-like isolate (closely related to a psychrotolerant strain found subsequently in a mine in Siberia) was the dominant iron oxidizer present. *L. ferrooxidans* was only detected in enrichment cultures using mine water inocula. The Norwegian AMD waters also contained significant numbers of acidophilic heterotrophs related to some

Table 3 Examples of mine water chemistries (all units are mg $\mathrm{l^{-1}}$, except pH)

	pH	$[Fe_{total}]$	$[Fe^{2+}]$	*[Al]*	*[Cu]*	*[Zn]*	$[SO_4]$
Coal mines							
Bullhouse (UK)	5.9	61	45	1.2	<1	<1	
Ynysarwed (UK)	6.2	160	140	20			460
Oatlands (UK)	5.5	287		0.97	<0.007	0.05	146
Sverdrupbyen (Norway)	2.7	179		27.5	0.168	1.3	1077
Metal mines							
Mynydd Parys (UK)	2.5	650	650	70	60	40	3100
Roeros (Norway)	3.7	6.7		4.3	11	3.76	
Wheal Jane (UK)	3.6	130	130	50	2	130	350
Cwm Rheidol (UK)	2.6–2.7			104–128	1.2–9.35	577–978	250
Sao Domingos (Portugal)	1.7	31 000	10 000				14 850
Iron Mountain (California)	1.5	2670	2470		293	58	14 000

Source: Data are from Johnson DB (2006) Biohydrometallurgy and the environment: Intimate and important interplay. *Hydrometallurgy* 83:153–166, and Nordstrom DK, Alpers CN, Ptacek CJ, and Blowes DW (2000) Negative pH and extremely acidic minewaters from Iron Mountain, California. *Environmental Science and Technology* 34: 254–258.

species (*Acidiphilium, Acidocella*, and *Acidisphaera*) that had previously been observed in acidic environments, and one (a *Frateuria*-like bacterium) that had not.

The importance of *At. ferrooxidans*-like bacteria in cooler (<20 °C) mine waters of pH 2–3 has also been supported at sites in other parts of the world. For example, biomolecular analysis (from clone libraries) of four AMD sites at the Dexing copper mine in the Jiangxi province of China found differences in the distribution of acidophiles with water pH. In the most acidic site (pH 1.5), *Leptospirillum* spp. (*L. ferrooxidans, L. ferriphilum*, and *L. ferrodiazotrophum*) were the dominant species in the clone library, while in pH 2.0 AMD *L. ferrodiazotrophum* was the single dominant species detected. In slightly higher pH (2.2) AMD, most clones recovered were related to *At. ferrooxidans*, while in the highest pH waters (3.0) most were related to the heterotrophic moderate acidophile *Acidobacterium*. Where mine waters have pH values of above 3, however, there is increasing evidence that moderately acidophilic iron oxidizers assume a more important role than *At. ferrooxidans*. The dominant iron oxidizer in AMD flowing from an underground coal mine in south Wales was found to be a *Thiomonas*-like bacterium, and similar strains (given the novel species designation *Thiomonas arsenivorans*) were isolated from an abandoned tin mine in Cornwall, England, and a disused gold mine (Cheni) in France. Other acidophilic Bacteria isolated from the Cornish site included *Acidobacterium*-like and *Frateuria*-like isolates, and an iron oxidizer related to *Halothiobacillus neopolitanus*. Further evidence of the importance of previously uncultured acidophiles in AMD has come from a study of acidic (pH 2.7–3.4) iron- and arsenic-rich water draining mine tailings at Carnoulès in France. The dominant bacteria found in clone libraries were betaproteobacteria, many of which were related to a *Gallionella*-like sequence previously reported in a chalybeate spa in north Wales. The sole *Gallionella* sp. that has been characterized (*Gallionella ferruginea*) is a neutrophilic iron oxidizer that grows best under microaerophilic conditions, and the circumstantial evidence for the existence of an acidophilic (or acid-tolerant) species of *Gallionella* is intriguing. Researchers also found evidence of SRB distantly related to *Desulfobacterium* in AMD at Carnoulès. SRB may also be found in sediments (and microbial mats) underlying AMD, though the pH in such sediments is frequently much higher than the AMD itself.

Microbiological studies of acid mine lakes in Germany have focused on phototrophic eukaryotes as well as acidophilic bacteria and have also examined how dissimilatory microbial reductive processes may be stimulated in order to ameliorate water acidity and immobilize metals. A survey of 14 acidic lakes in Lusatia (ranging in pH from 2.14 to 3.35, and conductivities from 690 to 4460 μS cm^{-1}) found a positive correlation between the relative numbers of the iron-oxidizing heterotroph *Fm. acidiphilum* and concentrations of aluminum. However, it was concluded that indicator groups of bacteria, rather than single species, were better correlated with different lake chemistries. Addition of organic carbon, nitrogen, and phosphorus to enclosed water columns in a pH 2.6 mine lake was shown to induce changes in both water chemistry and microbiology. Treatment of water resulted in increased microbial diversity, and SRB (*Desulfobacter* spp.) were among the microorganisms detected in the amended water columns.

One other important extremely acidic ecosystem that has been studied extensively is the Rio Tinto, a major river, some 92 km in length, located in southwest Spain (**Figure 3**). The source of the river is the Peña de Hierro (Iron Mountain) in the Iberian Pyrite Belt, and from there it flows though a large and historic area of copper mining (the Riotinto mines), eventually reaching the Atlantic Ocean at Huelva. Interestingly, even above the Riotinto mines, the river is acidic and enriched with metals, but this is very much accentuated as it flows through the (now abandoned) mining district. The river has a mean pH of about 2.2 and its distinctive red coloration derives from its soluble ferric iron content (~2 g l^{-1}). Primary production in the river is carried out by both photosynthetic and chemoautotrophic acidophiles. A study of the indigenous prokaryotes showed that >80% were Bacteria, and that Archaea accounted for only a relatively small proportion of cells. A variety of different iron oxidizers (*At. ferrooxidans, Leptospirillum* spp., *Fm. acidiphilum*, and *Fp. acidiphilum*) as well as the iron-reducing heterotroph *Acidiphilium* were identified. A geomicrobiological model involving cyclical oxidation of ferrous iron and reduction of ferric iron has been proposed to account for the remarkable chemical stability of the river ecosystem.

Acid Streamers, Mats, and Slimes

The most obvious and dramatic manifestations of microbial life in extremely acidic environments are macroscopic growths, referred to as acid streamers, slimes, and mats. Streamers may occur in flowing AMD streams inside and outside of abandoned mines; these have distinct filamentous morphologies and each filament may be more than a meter in length. Acid mats are denser in texture and are often found below growths of acid streamers. Acid slimes are thick, macroscopic biofilms that grow on moist surfaces of exposed rock faces. In addition, macroscopic filaments (or pipes) composed of acidophilic microorganisms may attach to, and suspend from, mine roofs and pit props. Where these are small-scale, they have been referred to as snotites, though larger structures have been described as microbial stalactites. Because of the macroscopic nature of acid streamer growths, they were among the first life-forms to be reported in extremely acidic environments (in 1938). Most of the early reports described acid steamers as being composed of bacteria embedded in a gelatinous matrix. The first attempts, in the 1970s, to characterize their component microorganisms used classical microbiological (cultivation-based) techniques. In the main, neutrophilic (chiefly spore-forming) *Bacillus* spp. were isolated from the acid streamers examined, leading to the erroneous conclusion that acid streamers were composed of neutrophilic heterotrophic bacteria that maintained circum-neutral pH within the macroscopic growths. In contrast, other researchers noted that streamers found in an iron/sulfur mine in Japan were able to catalyze the oxidation of both ferrous iron and sulfur, though some subsamples of streamers have very limited capacity to oxidize iron. *At. ferrooxidans*-like bacteria were isolated from these growths, leading the researchers to conclude that acid streamers were a mass of *At. ferrooxidans* embedded in a gelatinous matrix. However, it is only after biomolecular tools have been used to analyze acid streamers and related macroscopic growths that their true nature has been elucidated. This approach, coupled with the major advances made in the past two decades in techniques for isolating and cultivating acidophiles in the laboratory, has shown that streamer communities are both highly complex, and vary from site to site. Superficial similarities in their gross morphologies may mask completely contrasting microbial diversities.

One of the first intensive biomolecular examinations of acid slime (~1 cm thick) and snotite growths was carried out with materials collected from the Richmond mine at Iron Mountain, California. Microscopic examination showed that both growth forms were composed mostly of spirillum-shaped cells embedded in an extracellular polymeric matrix. Phylogenetic analysis based on 16S rRNA genes showed that most of the recovered sequences were novel, but related to known iron-oxidizing acidophiles. The single most dominant sequence recovered from slime growths was a novel strain of *Leptospirillum* (subsequently named *L. ferrodiazotrophum*). *L. ferriphilum*-related clone sequences were also identified. Other iron-oxidizing Bacteria identified were related to the Gram-positive actinobacteria, *Acidimicrobium*, and *Ferrimicrobium*, while sequences that affiliated with deltaproteobacteria (which includes anaerobic sulfate and iron reducers) were also detected, suggesting that microzones of low redox potential existed within the macroscopic growths. Archaeal genes were also amplified, and sequences related to *Fp. acidarmanus* were identified. Other archaeal sequences were, however, only distantly related to known Archaea.

In contrast to the Richmond mine slimes, acid streamer growths in less extremely acidic and cooler sites in north Wales (one an AMD stream at an abandoned copper mine and the other water in a chalybeate spa) were both found to be composed predominantly of betaproteobacteria. At the copper mine site, a single novel bacterium was the dominant prokaryote present, while this and a second betaproteobacterium accounted for 90% of bacteria (determined by the quantitative FISH technique) in the spa water streamers. A modified solid medium was developed to isolate the unknown bacterium from the copper mine streamers, and the betaproteobacterium isolate was shown to be the first representative of a novel genus (proposed name, *Ferrovum*) of iron-oxidizing chemoautotrophic acidophiles. The second (spa water) unknown species was identified as being most closely related to *G. ferruginea* (and a clone obtained from the Carnoulès mine; see 'Acid mine streams and lakes') but was not isolated. Although known species of acidophilic bacteria (*At. ferrooxidans, Acidiphilium, Acidocella, Thiomonas, Ferrimicrobium*, and *Acidobacterium*) were also isolated from the streamers, these were shown to be present in only relatively small numbers.

Another underground site that has been the focus of intense study is an abandoned pyrite mine (Cae Coch) located in northwest Wales. This mine is home to the most extensive and diverse macroscopic acidophile growths yet reported, with an estimated acid streamer biovolume of >100 m^3 alone, in addition to extensive slime biofilms and mats, microbial stalactites, and snotites. Although the temperature in the mine shows very little seasonal fluctuation (8.5 ± 1°C), other physico-chemical factors, including pH, dissolved oxygen, and concentrations of dissolved metals and other solutes, vary from site to site within the mine. This, together with the fact that the underground mine has been largely undisturbed for 90 years, has facilitated the colonization of different niches by different streamer/slime acidophilic communities. Using a combination of biomolecular and cultivation-based techniques, the Cae Coch streamer microflora has been shown to be very different to those of the AMD streams and ponds in which they bathe. All of the macroscopic growths were found to be composed of acidophilic bacteria (though protozoa and rotifers were also found in some locations), and the novel iron oxidizer *Ferrovum* was found to be the most abundant single organism present overall. Many of the Bacteria identified were well-known acidophiles (*At. ferrooxidans, Leptospirillum, Ferrimicrobium, Acidiphilium*, and *Acidocella*) while others (including *Frateuria*- and *Ralstonia*-like bacteria) were not. Included in the latter group was a *Sphingomonas* sp. that was detected by biomolecular methods, and also isolated in pure culture where it was shown to be an obligate acidophile, the first such species of *Sphingomonas* to have this trait. The fact that acid streamer communities can be very different from planktonic communities in the same ecosystem was also shown by a study of long (1.5 m) filamentous biofilms found in the Rio Tinto, Spain. Whereas the dominant microorganisms in the Rio Tinto water column are *Acidithiobacillus, Leptospirillum, Acidiphilium*, and archaea (Thermoplasmatales), the streamer-like growths examined were composed of gamma- and alphaproteobacteria; Gram-positive bacteria and betaproteobacteria were also detected in smaller numbers. As with the Cae Coch streamers, *Sphingomonas*- and *Ralstonia*-like bacteria were identified in clone libraries constructed from Rio Tinto streamers.

A more complex streamer/mat community in another mine site in southwest Spain (Cantareras) has been described recently (**Figure 7**). Both solar and chemical (mostly ferrous iron) energy drives primary production in an adit drainage channel at the site, and consequently acidophilic microalgae and chemoautotrophic iron-oxidizing Bacteria both thrive. The streamer growths that fill the ~100-m-long drain channel show distinct stratification (**Figure 7**). The surface layer is green due to the presence of *Zygnema, Chlamydomonas*, and other phototrophic eukaryotes that both aerate the anoxic mine water and provide organic carbon, which supports the growth of heterotrophic acidophiles. The lower layers are (in sequence) cream-brown, turquoise, and gray-black in color, and are almost exclusively bacterial. In contrast to most other acid streamer communities that have been described, heterotrophic (mostly iron-reducing) acidophiles dominate subsurface layers at Cantareras. This is particularly the case with the bottom mat layer, which is composed almost exclusively of *Acidobacterium*-like bacteria and novel strains of sulfate reducers. Complex biogeochemical cycling of iron (acting as both electron donor and electron acceptor) and sulfur in the Cantareras streamer community helps to sustain the highly diverse population of acidophiles that occurs there (**Figure 8**).

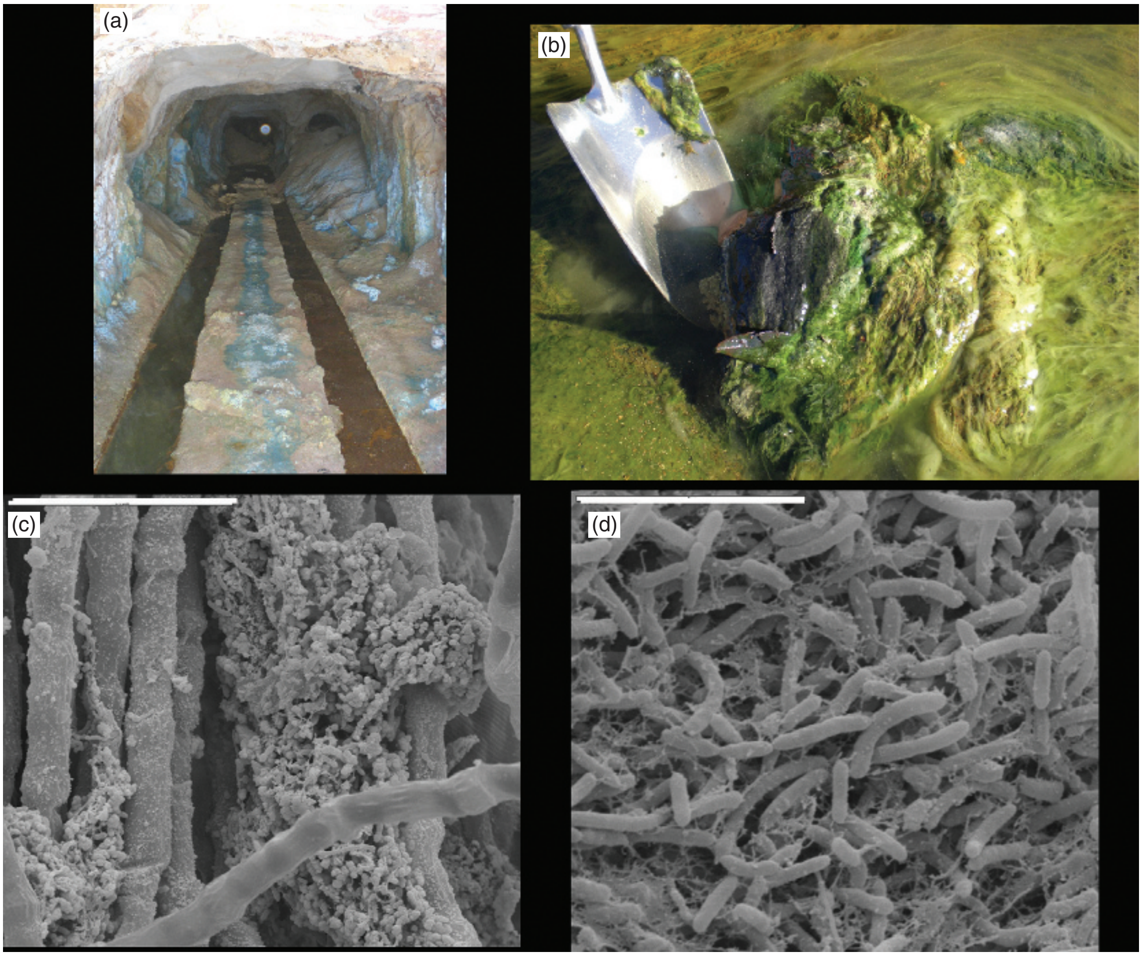

Figure 7 Acidophilic microbial communities in an abandoned copper mine (Cantareras, Spain): (a) acid mine drainage (AMD) channels draining the mine adit, showing deposition of copper salts on the adit walls; (b) stratified acid streamer and mat growths in the main drain channel; (c) scanning electron micrograph of the surface streamer layer, showing filaments of microalgae (*Zygnema*) and aggregates of bacteria (the bar scale represents 20 μm); (d) scanning electron micrograph of lower zone streamers, showing rod-shaped bacteria embedded in dehydrated exopolymeric material (the bar scale represents 5 μm).

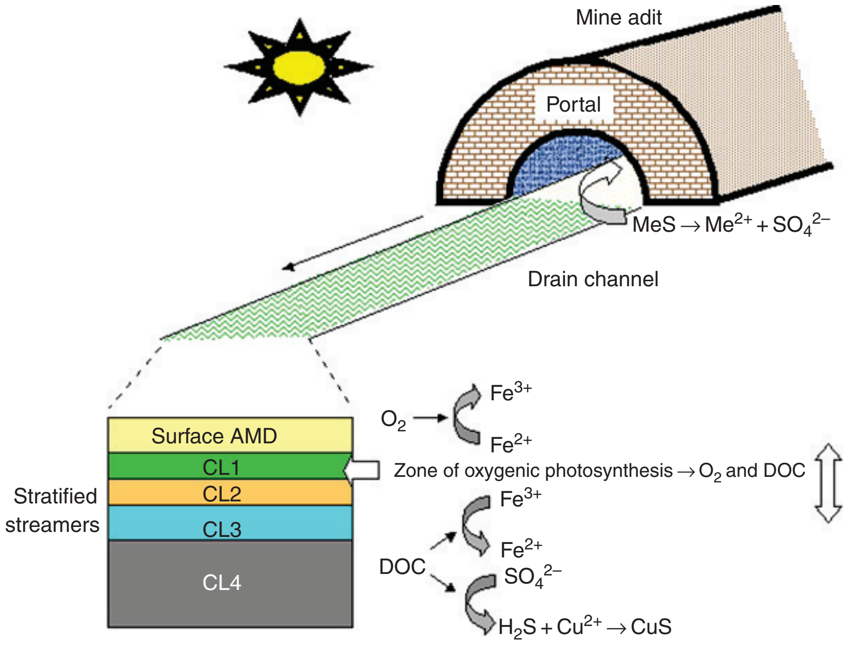

Figure 8 Proposed model of the biogeochemical cycling of iron and sulfur at the abandoned Cantareras mine. Dissolution of sulfide minerals in the exposed mine workings gives rise to a highly acidic, metal- and sulfate-rich effluent. The anoxic water draining the mine is oxygenated by photosynthetic acidophilic algae in the surface (CL1) layer of the acid streamer growths that develop immediately outside of the adit, which facilitates oxidation of ferrous iron in the surface AMD (catalyzed primarily by *Acidithiobacillus ferrooxidans*). Dissolved organic carbon (DOC) originating from photosynthetic and chemosynthetic primary producers serves as substrates for the (dominantly) heterotrophic bacteria in the deeper zone (CL2–4) streamer layers. Ferric iron is used as terminal electron acceptor in streamer layers CL2 and CL3, while in the thick CL4 layer sulfate is also used, resulting in the deposition of copper sulfide (CuS). The gradual buildup of ferric iron concentrations as the AMD flows through the channel results in the elimination of the microalgae, thereby removing the major primary production system that supports the streamer microbial community. Reproduced from Rowe OF, Sánchez-España J, Hallberg KB, and Johnson DB (2007) Microbial communities and geochemical dynamics in an extremely acidic, metal-rich stream at an abandoned sulfide mine (Huelva, Spain) underpinned by two functional primary production systems. *Environmental Microbiology* 9: 1761–1771, with permission from the publishers.

Acidophilic snotite-like biofilms have also been found in at least one other, very different, location. The Frasassi complex, located in central Italy, is a large and actively developing sulfidic cave, hosted in limestone rock. Large concentrations ($\sim$0.3 m mol l^{-1}) of hydrogen sulfide have been found in groundwater in deep sections of the cave system, and this gas has been shown to support the growth of sulfur-oxidizing acidophiles that grow in biofilms on the cave roof. Although the pH of the cave stream water is 7.0–7.3, droplets of liquid at the tips of the snotites have pH values of between 0 and 1, as a result of microbiological oxidation of sulfide, forming sulfuric acid. A bacterium related to *Halothiobacillus* was isolated from a snotite sample from Frasassi, together with clones related to *Acidithiobacillus* and *Sulfobacillus*. A later study showed that most of the clones obtained (65%) from a snotite sample from Frasassi were related to the mesophilic sulfur-oxidizing acidophile *At. thiooxidans*. The second most phylotype identified (31% of clones) was most closely related to *Am. ferrooxidans*, which is interesting as this is a moderately thermophilic acidophile that can oxidize ferrous iron but not reduced sulfur.

Outlook and Applications

Knowledge of the phylogenetic and physiological diversities of acidophilic microorganisms has expanded greatly in the past 25 years. Data from biomolecular studies of extremely acidic sites, however, suggest that a large number of acidophilic prokaryotes still await isolation and characterization. There is a great deal of interest in acidophiles, not only from the standpoint of understanding how these microorganisms can thrive in conditions that are hostile to most life-forms, but also due to their importance in environmental pollution (mine spoils and mine drainage waters) and in biotechnology (their central role in biomining and in removal of metals from contaminated soils). Significant research effort is currently concerned with finding acidophiles that can also tolerate other environmental extremes, such as temperature (above that of currently known thermoacidophiles) and salinity. There will doubtless be new opportunities to exploit existing and novel acidophilic microorganisms in future biotechnologies that will harness their unique abilities to thrive in conditions that are moderately or extremely acidic and mediate transformations of inorganic as well as organic chemicals.

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Extremophiles: Cold Environments

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Defining Statement
Discovery of Cold-Adapted Microbes
Evolution of Cold Adaptation
Molecular Basis for Cold Adaptation
Conclusion: A Model Habitat for Cold Adaptation
Further Reading

Glossary

barophilic Descriptor for cultured members of the domain Bacteria or Archaea that grow more rapidly under elevated hydrostatic pressures of the deep sea than at atmospheric pressure (also piezophilic).

barotolerant Descriptor for cultured members of the domain Bacteria or Archaea that grow under elevated hydrostatic pressures but more rapidly at atmospheric pressure.

cold-active General descriptor for enzymes that are catalytic or viruses that are infective at low temperature.

cold-adapted General descriptor for microorganisms, cultured or uncultured, that express traits enabling activity at low temperature.

cryopegs Ancient (>100 000 years) lenses of liquid brine of marine origin, found in deep layers of Arctic permafrost at temperatures of -10 to $-12\,^{\circ}$C.

enzymes Proteins that catalyze chemical reactions which otherwise would occur only slowly if at all; extracellular enzymes are held near the surface of the cell or released into the environment.

eutectic temperature Lowest temperature at which a mixture of salt and water can contain any liquid (about $-55\,^{\circ}$C for seawater); below it, liquid water converts to solid (ice) and dissolved salts precipitate; eutectophile refers to a microbe living near the eutectic temperature in a natural (saline) ice formation.

freezing point Temperature at which liquid water begins to convert to solid phase (ice); the freezing point of pure water (0 °C) is lowered by the presence of impurities (salt, organics).

lipid bilayer Critical component of the semipermeable membrane enclosing a microbial cell; when composed of unsaturated fatty acid-rich complexes, the lipid bilayer imparts greater flexibility to the membrane at low temperature.

mesophilic Thermal descriptor for cultured members of the domain Bacteria or Archaea that reproduce at a minimal temperature of $\sim$10 °C, optimal temperature near 37 °C, and maximal temperature of $\sim$45 °C.

oligotrophic Descriptor for nutrient-poor environments, where nutrients refer to organic substrates utilizable by heterotrophic microbes.

permafrost Perennially frozen soil or rock material, not subject to seasonal warming found in polar regions.

psychrophilic Thermal descriptor for cultured members of the domain Bacteria or Archaea that grow at a minimal temperature of 0 °C or lower, optimal temperature of 15 °C or lower, and maximal temperature of $\sim$20 °C (also stenopsychrophilic).

psychrotolerant Thermal descriptor for cultured members of the domain Bacteria or Archaea that grow at a minimal temperature of 0 °C or lower and maximal temperature above 20 °C (also eurypsychrophilic).

Defining Statement

Earth today is a cold planet, with over 80% of its biosphere at temperatures of $\leq 5\,^{\circ}$C and 10–20% of its surface frozen. Widely diverse microbes have evolved specific molecular, cellular, and extracellular adaptations to enable their essential roles in the biogeochemical cycles of the planet.

Discovery of Cold-Adapted Microbes

Earliest Observations and Terminology

The earliest known report of microbial life in a cold environment dates back to the fourth century BC and the writings of Greek philosopher Aristotle who made observations of what later proved to be photosynthetic Eukarya ('red algae') that turned snow to a reddish color.

More than two millennia later in the nineteenth century (1887), the German scientist Forster described the ability of a bioluminescent bacterium, derived from fish preserved in the cold, to reproduce at 0 °C. In the twentieth century (1902), the term 'psychrophile' for cold loving was introduced by Schmidt-Nielsen to describe such microorganisms.

Over the next 60 years, the term psychrophile continued to be in use to describe cold-adapted microbes according to the ability to reproduce in the cold, regardless of the upper temperature limit for growth. That limit, with few exceptions, fell above room temperature, thus overlapping with the thermal category of mesophiles. These microbes today are called psychrotolerant (or psychrotrophic), with the descriptor psychrophilic reserved for organisms that fit the more precise definition provided by the American marine microbiologist Morita (in 1975), based on cardinal growth temperatures: minimal temperature ($T_{\min}$) of 0 °C or lower, optimal temperature (T_{opt}) of 15 °C or lower, and maximal temperature ($T_{\max}$) of about 20 °C (**Figure 1**). By this definition, which provided much needed clarity at the time and remains in wide use today, the first true psychrophile was discovered by Tsiklinsky as part of a French expedition to Antarctica (1903–05). Another half-century would pass before the abundance and functional roles of true psychrophiles in cold environments would be appreciated. First, microbiologists had to learn about the sensitivity of cold-adapted microbes to room temperature (even to unchilled pipets) during isolation procedures. Eventually, after many enrichment studies using 4–6 °C (convenient refrigerator temperature) and reports of predominantly psychrotolerant isolates, came the understanding that the temperature of initial enrichment influences the thermal nature of the resulting isolates: enrichments near the freezing point (e.g., at −1 °C for marine samples) are more likely to yield psychrophilic than psychrotolerant bacteria.

Morita expected that future adjustments to his definition of psychrophily, based on new isolates obtained by paying attention to detrimental (and influential) temperatures during isolation procedures, would be in the direction of lower cardinal temperatures. Microbes with considerably lower cardinal growth temperatures, called extremely psychrophilic, have been reported in the last decade, particularly the current record holders for low-temperature growth, *Psychromonas ingrahamii* ($T_{\min} = -12\,°\text{C}$, $T_{\text{opt}} = 5\,°\text{C}$, $T_{\max} = 10\,°\text{C}$) and *Colwellia psychrerythraea* strain 34H ($T_{\min} = -12\,°\text{C}$, $T_{\text{opt}} = 8\,°\text{C}$, $T_{\max} = 18\,°\text{C}$) from subzero Arctic sea ice and sediments, respectively. Measurements of microbial metabolic activity at very cold temperatures (down to at least −20 °C) in natural ice formations, where measuring a reproductive rate is methodologically challenging, has led to introduction of the term eutectophile for the most cold-adapted microbes, living in ice near the eutectic temperature with only nanometer-scale films of liquid water available.

Because cardinal growth temperatures cannot fully capture the adaptability of a microorganism to its environment and are unavailable for the vast majority of microbes in nature that evade cultivation, the term psychrophilic (and psychrotolerant) remains most useful for categorizing cultured members of the domains of Bacteria and Archaea. Cold-adapted is used very generally to describe microorganisms, cultured or uncultured, that express a recognizable adaptation to low temperature, while cold-active is often reserved for enzymes and viruses with catalytic or infective activity at low

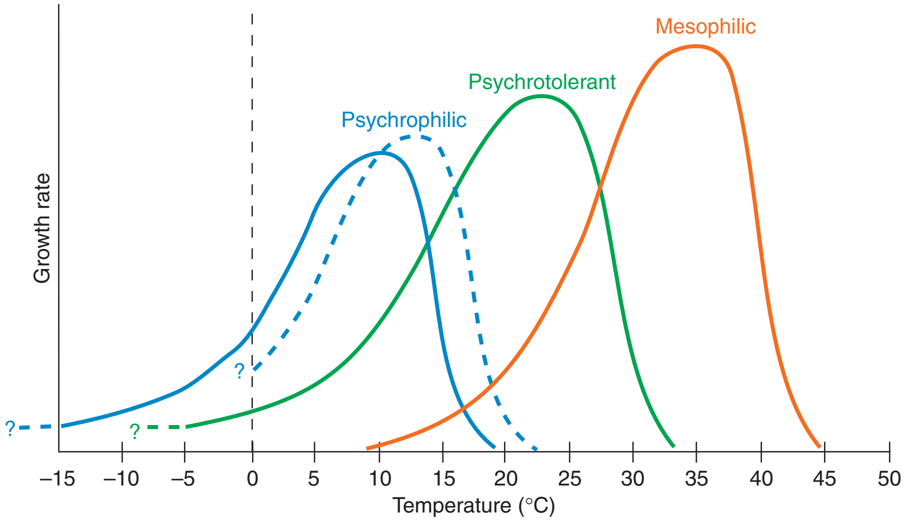


Figure 1 Schematic depiction of bacterial growth rate as a function of temperature (at atmospheric pressure) for a psychrophilic, psychrotolerant, and mesophilic microbe. The dotted line depicts the psychrophilic response when grown under elevated hydrotstatic pressure.

temperature. Other terms based on cardinal growth temperatures are recently in play, particularly stenopsychrophilic and eurypsychrophilic (comparable in operational meaning to psychrophilic and psychrotolerant, respectively), in an attempt to recognize that psychrotolerant microbes are not simply tolerant of the cold (some mesophiles and thermophiles can tolerate the cold) but also adapted to it, in spite of higher T_{max} for growth than psychrophiles.

What constitutes a cold temperature is also subject to perspective. Combining cardinal growth temperatures for psychrophiles and some key environmental temperatures yields the following set of descriptors. Moderately cold is 15 to 5 °C; that is, from the upper T_{opt} for psychrophilic growth, which is also the average temperature of the surface of the Earth, down to the (upper) temperature of 80% of the biosphere. Cold is 5 to −2 °C, the approximate freezing point of seawater, while very cold is below −2 °C. Extremely cold is below −12 °C, the current lowest T_{min} for microbial growth in culture and the temperature of the Earth's near-million year-old permafrost in polar regions. Cold in the term cold adaptation remains broadly defined by any temperature 15 °C or lower.

Exploration of the Cold Deep Sea

The cold deep sea, as the volumetrically dominant and most persistently cold environment on Earth (over geologic time), has provided an important natural laboratory for studying and advancing understanding of cold adaptation in the microbial realm. Although its temperature is always above the freezing point of seawater and thus not as thermally extreme as most frozen environments, the cold deep sea is considered extreme for other reasons: its elevated hydrostatic pressure, which increases linearly at 10 atm (=1 MPa) per 100 m increase in water depth, and its typically oligotrophic state. The search for psychrophiles in the cold deep sea has often been coupled with the search for barophiles (also known as piezophiles), pressure-adapted microbes that grow more rapidly under elevated hydrostatic pressures than at atmospheric pressure when adequate nutrients in the form of organic substrates are available. The focus of these searches has usually been the heterotrophic bacteria.

Scientific exploration of oceanic life, in general, began with a series of deep-sea expeditions at the end of the nineteenth century (study of the productive surface layers and sea ice would come later). Until the discovery of deep-sea hydrothermal vents toward the end of the twentieth century (in 1977), the deep ocean was understood to be uniformly cold, the temperature not exceeding about 5 °C (except in the deep Mediterranean and Sulu seas where temperatures reach about 15 °C). Yet, early expeditions had no facilities for incubating samples shipboard at such low temperature. The first opportunities to discover cold-adapted microbes from the vast, cold deep sea were thus thwarted by lack of refrigeration. Remarkably, the early French explorer Certes was able to test for pressure-adapted microbes from the deep sea in the 1880s, even if cold adaptation was beyond reach.

More than a half century later, American marine microbiologist ZoBell began the study of deep-sea microbes under both *in situ* pressure and temperature, documenting with Morita in 1957 the first psychrophilic barophiles. Although these cultures were lost to future study, similar efforts by several groups beginning from the 1970s eventually yielded sizeable culture collections of psychrophilic barophiles; indeed, all barophiles cultured from the cold deep sea are also psychrophilic. The synergistic effects of temperature and pressure on microbial growth (or other activities) have not been fully explored, but for several psychrophilic bacteria their cardinal growth temperatures can be shifted upward by incubating under higher pressure (**Figure 1**). When the growth responses of deep-sea bacteria have been examined according to a matrix of three parameters – temperature, pressure, and salinity – salt concentration is also observed to shift cardinal growth temperatures, though the direction of the shift is variable and strain-dependent. Microbiological exploration of the cold deep sea has thus raised general awareness that cold adaptation must be understood in relation to other parameters and not exclusively to temperature.

Not least of other parameters is the concentration of available energy sources or organic substrates in the case of the heterotroph. For heterotrophic psychrophilic barophiles the barophilic trait depends upon available substrate concentration: at low substrate concentrations, growth rate is similar under both atmospheric and elevated pressures (barotolerant, within the pressure range for growth), while at high substrate concentrations, growth rate is higher under elevated pressures than at atmospheric pressure (barophilic). When substrates are in adequate supply in the cold deep sea, for example, from the hydrolysis of freshly deposited organic detritus, psychrophilic barophiles will outcompete other cold-adapted microbes that may be present. Genetic work with pressure-adapted psychrophiles indicates that membrane proteins involved in substrate uptake are upregulated under elevated pressures when provided with sufficient substrate.

Somewhat analogous work with marine psychrophiles from shallow cold waters, considering only temperature and substrate concentration, indicates that the lower the temperature, the higher the required substrate concentration to achieve a comparable growth (or oxygen respiration) rate. Low temperature or viscosity-driven reduction of the diffusive flux of various solutes to the cell is insufficient to account for this increase in the required substrate concentration threshold. Resolving the issue is important to understanding why, at least at times, the microbial role in biogeochemical cycles of surface polar waters appears limited until sufficient organic solutes accumulate. Although gene expression work that may help to explain this phenomenon remains to be conducted, the recent whole-genome analyses of several heterotrophic psychrophiles reveals an apparent ability to store substrate reserves intracellularly, a potential means around the problem for the individual cell. Also awaiting a gene-based explanation is the repeated observation that cold temperature favors higher bacterial growth efficiency: a greater fraction of the organic carbon consumed in the cold goes to new biomass (gain to the food chain) than to respiration (loss as carbon dioxide). Warming temperatures in polar waters are thus expected to shift the role of cold-adapted microbes in the cycling of organic carbon to increased remineralization to carbon dioxide.

Exploration of Other Low-Temperature Environments

Many other cold environments have been explored over the past several decades for cold-adapted microbes. Moderately cold to cold environments include a wide range of aquatic and sedimentary environments, both marine and freshwater (rivers, lakes, and subsurface aquifers), terrestrial soil environments of varying degrees of desiccation, and numerous surfaces that support microbial biofilms, from moist rocks in caves and mines to fluid-bathed tissues of vertebrate and invertebrate animals that live in the cold. Unlike the cold deep sea, many of these environments experience fluctuating temperatures (and other parameters) on a seasonal and diurnal basis. Exceptions with stably cold temperatures include animal-associated microbial environments, where the animal host spends its life history in cold water, as well as the many deep subglacial lakes of Antarctica (over 150 have been discovered) with stable temperatures just above the freezing point, given separation from the lower atmosphere and solar radiation by kilometers of glacial ice. With the possible exception of subglacial lakes, which await direct exploration (drilling efforts have only reached ice accreted above the water line), all of these cold environments have in common their successful colonization by cold-adapted microbes, which then play active if not dominant roles in cycling the inorganic and organic materials within them. Based on classical chemostat studies pitting cold-adapted microbes with different cardinal growth temperatures against each other at constant low temperature, the more psychrophilic organisms are the dominant players.

In contrast are the environments that experience very cold to extremely cold temperatures, including the upper atmosphere where viable microbes have been found associated with microscopic particles and, of course, the major types of ice formations on Earth – freshwater snow and glacial ice (formed from long-term compaction of snow), lake and river ice, polar sea ice, and frozen soils. Except for sea ice (see below) and possibly lake ice (understudied), frozen environments in general can be viewed as preserving an often cosmopolitan suite of microbes, largely inactive in the cold, rather than as actively colonized by cold-adapted microbes with all the attendant successional and adaptive responses. At extremely cold temperatures, the primary limitation to the latter scenario is the absence of sufficient water in the liquid phase. All ice formations on Earth derive from source waters containing impurities of one kind or another that depress the freezing point (especially inorganic salts), so they retain at least some liquid water. Only those that may drop below their eutectic temperature, for example, high altitude glacial ice on Antarctica, become completely desiccated.

Some of these frozen environments experience seasonal and/or diurnal temperature swings, which intermittently relieve the limitation of insufficient liquid water. They can then support highly productive microbial ecosystems. An example is sea ice, which during spring and summer seasons, with near-continuous sunlight and seawater flushing its base with nutrients, develops algal and microbial communities visible to the naked eye as strongly discolored ice (**Figure 2**). These communities contribute 25% or more of total primary production in the Arctic with consequent effects on secondary production (the transformation and consumption of this biomass) and higher trophic levels. Sea ice (like lake and river ice), however, is not a stable environment, melting by late summer before reforming in fall. An exception is multiyear sea ice in the Arctic Ocean that until recently could survive 8–10 melting seasons before circulating out of the Arctic into melting Atlantic waters. Climate-driven declines in multiyear (and first-year) sea ice, which had been averaging about 10% per decade since satellite coverage began in the 1970s, recently accelerated beyond all model predictions. This particular frozen environment may soon represent a lost opportunity for the study of cold adaptation.

The upper layers of soil in alpine and polar regions also experience regular and wide fluctuations in temperature seasonally, from warm (>20 °C) to extremely cold, as well as climate-driven warming. Even during moderately cold periods, microbial activity increases such that the environment becomes a source of greenhouse gases like carbon dioxide and methane, rather than a sink. In alpine soils, an insulating snow cover promotes substantial microbial activity through the winter. The deeper frozen layers (>50 m) of polar soils removed from atmospheric and solar influences, however, have been permanently frozen (permafrost) in the temperature range of −10 to −12 °C for close to a million years in some Siberian

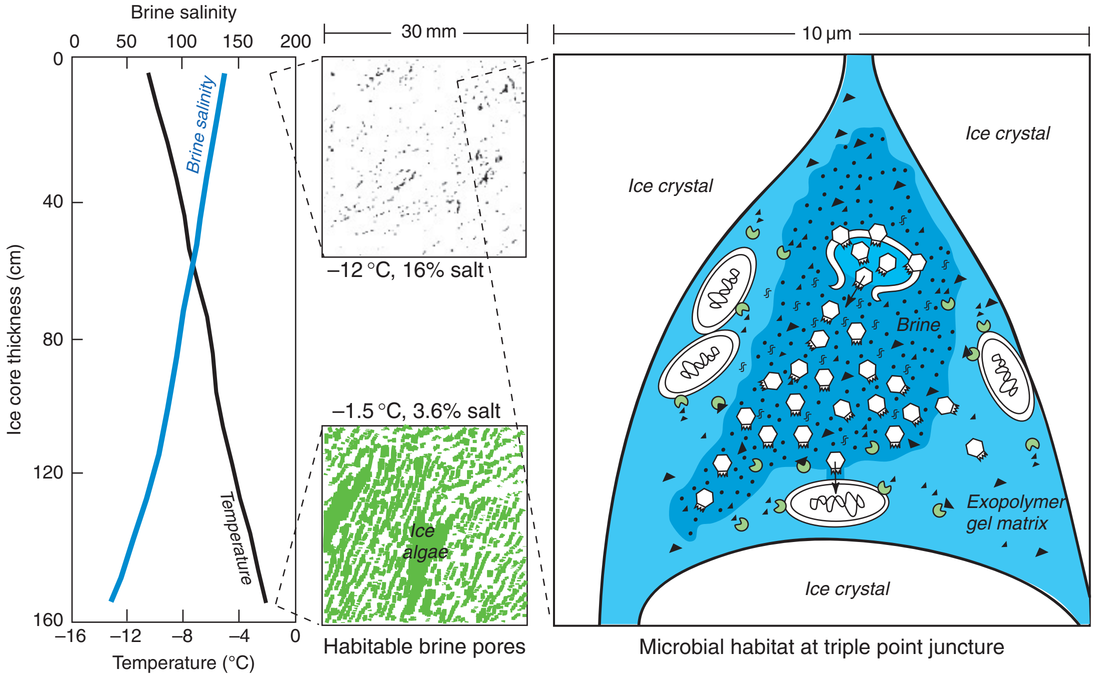


Figure 2 Schematic depiction of some characteristics of sea ice. At left are vertical gradients, in temperature and in salinity of liquid inclusions, that develop in winter as temperature of the overlying atmosphere drops but underlying seawater remains near freezing point. Middle panels depict relative size of brine pores in a very cold section of ice versus bottom ice with larger channels flushed by seawater that will support an ice-algal bloom in spring. At right is an enlarged schematic of very cold brine at the juncture of three ice crystals, depicting microbes embedded in a gelatinous matrix of exopolymers, brine, and organic substrates concentrated in the interior, and extracellular enzymes hydrolyzing the substrates. Also shown are proposed cold-active viral enzymes at work, successful infection and viral reproduction, lysis of host and release of free DNA and new viruses, potential agents of horizontal gene transfer.

locations. The typical diversity of soil microbes that have been recovered in culture from permafrost, including aerobes, anaerobes, heterotrophs, sulfate reducers, and methanogens, may represent some of the oldest viable forms available to study. Wedged between deep layers of permafrost are cryopegs, recently discovered lenses of very old unfrozen water kept liquid by high salt concentration. Whether these very cold brines are life preserving or actively colonized remains to be determined.

In the upper atmosphere, high-altitude glacial ice and snow that covers Greenland and Antarctica, and Arctic winter sea ice (and its overlying snow), microbes experience extremely cold temperatures, sometimes approaching or reaching the eutectic of −55 °C (for seawater). The thermal gradients inherent to glacial and sea-ice environments (**Figure 2**) provide natural laboratories to examine the question of the lower temperature limits for microbial growth, activity, and survival. To date, studies of such environments and of artificially produced ices at extreme temperatures suggest that cellular reproduction may be limited to about −20 °C, metabolic activity to about −40 °C, and survival to the lowest temperatures yet to be tested (−196 °C in liquid nitrogen). These general guidelines, however, are subject to change, especially as the field of Astrobiology stimulates increased experimentation under extremely cold conditions.

Early in the study of this varied array of low-temperature environments, a general paradigm emerged: stably cold environments tend to support a greater (culturable) community of psychrophilic microbes, while those with temperatures that fluctuate, especially above the $T_{\max}$ of psychrophiles, tend to support a greater community of psychrotolerant (eurypsychrophilic) microbes. The implication was that psychrophily requires a stably cold environment to evolve. Although this paradigm appeals to common sense, much of the early data supporting it relied upon enrichment temperatures that would have favored psychrotolerant microbes. When more stringent enrichment conditions are used (e.g., −1 °C for saline environments), temperature-fluctuating environments that previously yielded mainly psychrotolerant isolates yield a predominance of psychrophiles instead. Psychrophiles have also been observed to reestablish dominance in an environment in a relatively short period of time (days), once fluctuating temperatures have stabilized at a cold temperature. Furthermore, the sea-ice environment, which has always consistently yielded greater numbers of psychrophiles, is an ephemeral one, with inhabitants released during the summer melt period to seawater that then warms under 24 h solar radiation. From initial encasement during ice formation in fall through the winter, spring and summer seasons, sea-ice microbes can experience a temperature fluctuation of more than 40 °C, from −35 in winter to 6 °C (or higher) in melt water. The corresponding fluctuations in other parameters, especially salt concentration (**Figure 2**), add osmotic and other stresses to the thermal swing. Although psychrophiles have long been considered the more sensitive of cold-adapted microbes, in large part because some express narrow temperature ranges for growth (unlike that shown in **Figure 1**), environmental robustness is a matter of perspective influenced by considering other parameters. What constitutes an ideal habitat for cold adaptation needs to be reconsidered.

Influence of Astrobiology

The ongoing and planned exploration of other bodies in our solar system for evidence of past or present life opens a new chapter in the history of studying cold-adapted extremophiles. Even the extremely cold environments of Earth are thermally moderate relative to the extraterrestrial sites targeted for exploration. For example, the average surface temperature of the desiccated soils on Mars is about −55 °C, coincidentally the eutectic temperature for Earth's seawater. Where the surface temperature is warmer, not even nanometer-scale films of liquid water are available and radiation intensity (in the absence of a protective atmosphere as on Earth) would be prohibitive. The average temperature of the deeply frozen surface of Europa, the Jovian moon believed to harbor beneath its ice cover a global ocean larger than Earth's, is about −160 °C. Such extremely cold ice formations are not found on Earth, invoking the need to study forms of ice generated in the laboratory. Initial studies of the reactions of a known psychrophile to flash freezing, as might be experienced when Europan seawater rises into cracks of its extremely cold ice cover, suggest the importance of organic (sugar-rich) exopolymers in buffering cells against fatal damage from ice crystals and even enabling the completion of enzymatic reactions begun prior to freezing. Because the presence of exopolymers are also known to alter the microstructure of saline ice in readily detectable ways, similar effects in extraterrestrial ices may constitute a recognizable biosignature.

Although space missions first access only extremely cold surfaces, the subsurface environments of both Mars and Europa, hidden from damaging radiation, hold greater promise for life. They are expected to be more moderate in temperature, favoring liquid water (perhaps in analogy to the brine layers of deeply buried permafrost), and to offer potential energy sources for chemolithotrophic life forms, for example, via the exothermic water-rock reaction known as serpentinization. In Earth's deep sea at a mid-Atlantic site called Lost City, serpentinization is known to yield hydrogen and methane in support of luxurious archaeal biofilms and mats. The study of cold-adapted Archaea and chemolithotrophs in general is in its infancy, relative to the heterotrophic bacteria.

Evolution of Cold Adaptation

Glaciation Periods on Earth

The temperature of the early Earth and its ocean is actively debated, but marine geological evidence points to a very hydrothermally active and thus warm ocean. The first hypothesized period of planetary-scale chilling or glaciation does not occur until about half-way through Earth's history at 2.2 billion years ago during the Proterozoic era. Less than a billion years ago, Earth is believed to have experienced a severe freezing episode resulting in what has been called 'Snowball Earth'. Since then, the planet has experienced a series of glaciation events, not always global in scale, eventually leading to the glacial/interglacial periods of recent Earth history. Their periodicity is estimated in tens of thousands of years. In between each major ice age, the planet is believed to have been completely free of ice with an average temperature above that permissive of a psychrophilic life style.

Unless the deep sea remained sufficiently cold to provide refuge to a stock of psychrophiles, a difficult hypothesis to test, psychrophilic microbes likely evolved more than once during Earth's history. The implication is that the evolutionary steps between psychrotolerance and psychrophily must be accommodated by the time available between glaciation periods. In addition to vertical gene transfer from an ancestor (inherited beneficial gene mutations), horizontal gene transfer (e.g., mediated by viruses) may have played important roles in achieving these steps. Leading the way to tests of this hypothesis are phylogenetic analyses of extant microbes, cultured and uncultured, comparative genomic evaluations of psychrophiles and other thermal classes of microbes, and experimentation with horizontal gene transfer in the cold.

Phylogeny of Cold Adaptation

The now classic 16S rRNA gene-sequencing approach to deducing relationships among organisms yields a universal tree of life on which known psychrophilic and psychrotolerant microbes can be located. Use of a phylogenetic tree originally designed to highlight hyperthermophilic genera of Bacteria and Archaea (those that grow at 90 °C or higher) for this purpose emphasizes the late arrival of cold adaptation among extant organisms (**Figure 3**). It also indicates the slightly deeper branching of groups containing only psychrotolerant members, reinforcing the expectation that psychrophiles evolved from psychrotolerant strains.

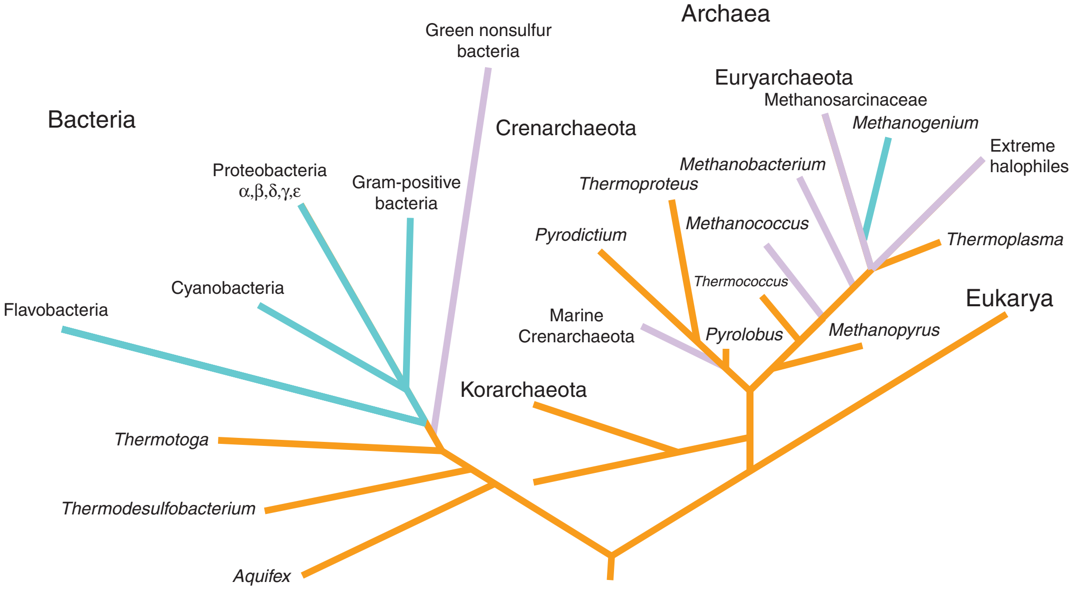


Figure 3 Universal phylogenetic tree of life based on 16S rRNA sequences, emphasizing the domains of Bacteria and Archaea. Orange branches indicate hyperthermophiles that grow at $\geq$90 °C; purple branches, groups that contain known (cultured) psychrotolerant strains; and blue branches, groups that contain known psychrophiles. Note that the (uncultured) marine Crenarchaeota are colored purple because degree of cold adaptation is not known.

Most of the major branches within the domain of Bacteria, except those unique to thermophiles, contain psychrophilic members, including all five groups of the *Proteobacteria*, aerobes and anaerobes alike, the *Cytophaga-Flavobacteria*, the *Cyanobacteria*, and the Gram-positive bacteria. Some genera of the gamma-Proteobacteria, in particular *Moritella* and *Colwellia*, are comprised mainly or exclusively of psychrophiles. The Green nonsulfur bacteria, however, contain only psychrotolerant isolates so far.

In the domain of Archaea, only a single culture-authenticated psychrophile is known, the methanogen *Methanogenium frigidum*, isolated from an Antarctic lake. Its position within the archaeal domain of the tree also indicates a later evolutionary arrival (**Figure 3**). The marine Crenarchaeota have a somewhat earlier branching position. Members of this archaeal group often dominate numerically in the cold deep sea and occur in many of the polar waters and sediments that have been examined, but only one of their members has been brought into culture (from an aquarium sample enriched at room temperature). It is not cold-adapted. A well-studied crenarchaeal symbiosis with a sponge host that dwells in cold waters clearly suggests cold adaptation, but whether psychrophilic or psychrotolerant awaits a cultured isolate. Because the marine Crenarchaeota that inhabit the cold deep sea are believed to be involved in the nitrogen cycle, especially the process of nitrification (microbial oxidation of ammonia to nitrite and nitrate) which generates the inorganic nitrogen required by primary producers in surface waters, they are targets of intensive study.

Given that the study of cold adaptation in the microbial realm has historically centered on heterotrophic bacteria, in large part because these organisms are more readily brought into culture than chemolithotrophs or Archaea in general, conclusions from the depicted phylogenetic tree (**Figure 3**) should be drawn with caution. As more microbes are brought into culture and shown to be cold-adapted, the branching patterns evident today may change. Stable isotope (and other) probing techniques that allow recognition of microbial activity under different temperatures in the absence of cultivation, yet coupled to a sequencing identification, may also bring new information to the tree.

Genetic Mechanisms

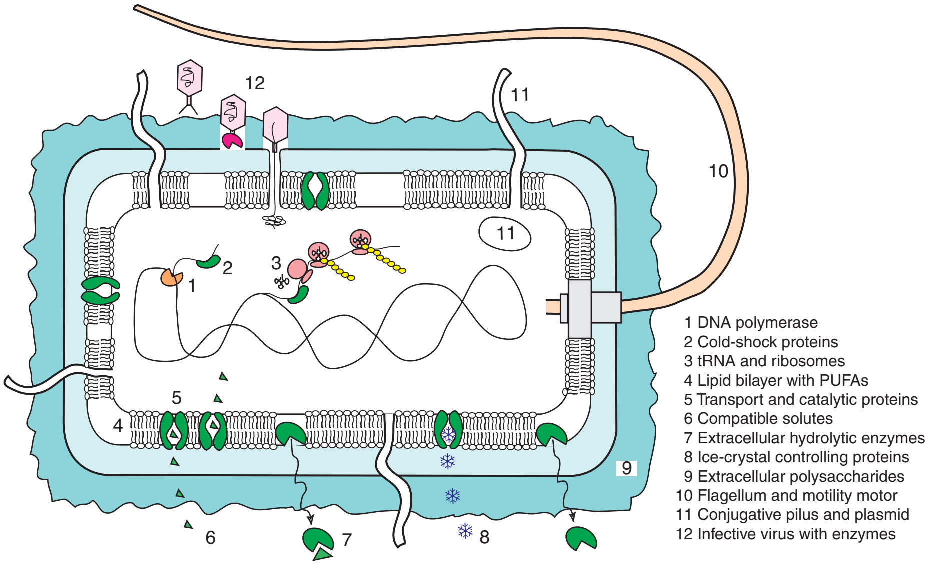
Genetic mutation as a means to cold adaptation is evident from studies of the molecular interactions inferring enzymes with catalytic ability in the cold and in comparative analyses of whole-genome sequences from related organisms with different cardinal growth temperatures. In the former case, site-directed mutagenesis and related approaches indicate that, depending on the enzyme and often its size, anywhere from a single amino acid change to numerous amino acid substitutions or chemical alterations can explain the gain (or loss) of cold activity. In the latter case, and despite an oft-cited idea that only a critical subset of an organism's enzymes need be cold-active for it to function as a psychrophile, results of comparative genome studies for both Archaea and Bacteria suggest otherwise. Significant amino acid replacements were observed in over 1000 genome-deduced proteins from the psychrophilic methanogen, *M. frigidum*, relative to proteins from other methanogens covering a range of temperature growth optima, from 15 to 98 °C. When the whole proteome of *C. psychrerythraea* 34H and three-dimensional architectures of its proteins were compared to nearest mesophilic neighbors with available genome sequences, the same general observation was made. In both cases, the interactions and locations of polar and charged amino acids (in particular serine, histidine, glutamine, and threonine) in protein tertiary structures appeared influential in imparting psychrophily.

In other analyses of the whole genomes from the several psychrophiles that have been sequenced so far, specific proteins and other features known from previous culture work to impart cold adaptation (discussed below) have been documented. A new genomic finding, not widely evident from prior culture work, is the prevalence of virally delivered genes in psychrophiles along with the presence of prophage (viruses residing benignly in the bacterial genome and thus implicated in gene transfer). That virally mediated horizontal gene transfer has played an important role in the evolution of psychrophily appears inescapable, although other forms of gene transfer also need to be considered (transformation by direct uptake of free DNA and plasmid exchange by conjugation between cells; **Figure 4**), particularly since the transfer of plasmid DNA via conjugation between mesophilic and psychrophilic bacteria has been demonstrated.

In the same time frame that genomic analyses of psychrophiles have become available, the study of cold-active virus–host systems in culture has been renewed. While work over a half-century ago had documented infectivity at 0 °C, the environmental and evolutionary implications had only rarely been pursued. Recently, several promising cold-active virus–host systems have been obtained by a number of researchers and the lower (known) temperature limit for infectivity has been pushed to −12 °C (and 16% salt) in simulated sea-ice brines. The goal of demonstrating active horizontal gene transfer under realistic environmental conditions, whether or not mediated by viruses, as means to evolve cold adaptation remains for the future.

Molecular Basis for Cold Adaptation

As the temperature of an aqueous solution decreases, for example, from 37 to 0 °C, its viscosity more than doubles, slowing solute diffusion rates, while chemical reaction rates (also influenced by viscosity) decrease exponentially. The cytoplasmic membranes and enzymes of mesophilic microbes tend to rigidify under these conditions and lose function. Protein folding is impaired and nucleic acids assume secondary or super-coiled structures that interfere with their proscribed activities. A hallmark of microbial cold adaptation at the molecular level is thus to retain sufficient flexibility in its macromolecules such that essential functions can go forward in spite of the challenging effects of low temperatures. When arguably nonessential functions, for example, motility, can also go forward, the microbe becomes even more competitive in the cold. The lower temperature limit for bacterial motility, −10 °C (in a high sugar solution), is held by an extreme psychrophile, *C. psychrerythraea* 34H. Enough direct research on psychrophiles and comparative studies of psychrotolerant and mesophilic bacteria has been accomplished in recent decades to identify key components and aspects of a cell that impart cold adaptation (**Figure 4**).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3 tRNA and ribosomes
4 Lipid bilayer with PUFAs
5 Transport and catalytic proteins
6 Compatible solutes
7 Extracellular hydrolytic enzymes
8 Ice-crystal controlling proteins
9 Extracellular polysaccharides
10 Flagellum and motility motor
11 Conjugative pilus and plasmid
12 Infective virus with enzymes

Figure 4 Simplified schematic of a bacterial cell depicting some of the known components and processes linked to cold adaptation (see text).

Membrane Fluidity

The cellular membranes of cold-adapted microbes must remain flexible enough under rigidifying temperatures to facilitate their essential functions in the transport of nutrients and metabolic byproducts and the exchange of ions and solutes critical to maintaining intracellular integrity. The cold-adapted cell accomplishes this feat by fine-tuning the composition of its membrane lipid bilayer (**Figure 4**), introducing steric hindrances that change the packing order of the lipids or reduce interactions between them and other membrane components. The genome must encode the ability to produce a flexible membrane in the first place (adaptation), as well as to adjust membrane components on the short-term (acclimation). The list of specific alterations known to enhance flexibility in the cold is extensive, if sometimes strain-specific, but typically includes producing a higher content of unsaturated (fewer double bonds between carbon atoms), polyunsaturated, and branched and/or cyclic fatty acids in response to cold. In some cases, shortening the length of the fatty acid can enhance flexibility, while in others, changing the content or size of the lipid head groups helps. Genes for enzymes involved in polyunsaturated fatty acid (PUFA) synthesis are clearly present in psychrophilic genomes, although without tests of their cold-active nature, such findings are not definitive for cold adaptation (some mesophiles also contain them).

Other components of the lipid bilayer include membrane proteins and carotenoid pigments. How membrane proteins interact with lipids affects both the active and passive permeability of the membrane, important for controlling the exchange of ions and organic solutes (**Figure 4**). The genome of *C. psychrerythraea* 34H contains numerous gene families involved in the transport of compatible solutes, low-molecular-weight organic compounds (often sugars or amino acids and their derivatives) that accumulate to high intracellular levels under osmotic stress, and are compatible with the metabolism of the cell. They help to maintain cellular volume and turgor pressure and protect intracellular macromolecules in the face of changing salt concentrations exterior to the cell, as occurs in sea-ice brines when the temperature drops (**Figure 3**). The cold-adapted membrane thus needs to be flexible, especially in very cold environments, to permit both the uptake of energy-yielding substrates and compatible solutes, yet impermeable enough to prevent excessive passive exchange; adjusting the protein components of membranes can help. The sensitivity of some membrane proteins to temperature-driven conformational changes appears to provide a thermal sensor that results in the upregulation of genes involved in subsequent membrane adjustments, making life in a temperature gradient imminently feasible. As temperatures approach $T_{\max}$ for growth, cold-adapted microbes must also be able to keep their membranes sufficiently stable (inflexible) to avoid cell leakage and death by lysis. In some cases, adjusting pigment content appears to accomplish this goal.

Cold-Active Enzymes

The exponential drop in chemical reaction rates brought on by decreasing temperature highlights the impressive evolutionary development of all manner of enzymes with high catalytic activity in the cold. Complimenting the known membrane adjustments to achieve flexibility in the cold are those expressed by cold-active enzymes, including both essential intracellular enzymes required for nucleic acid and protein synthesis in the cell, membrane permeases for active solute transport and alteration, and extracellular enzymes released to perform hydrolytic functions in the environment (**Figure 4**). As with achieving a more flexible membrane against the cold, high enzymatic activity at low temperature involves creating greater molecular flexibility than that observed in enzymes active at warm temperatures. It also involves trade-offs; although not a firm rule, for many cold-active enzymes the very traits that impart cold activity also make them unstable at higher temperature. Unlike membrane adjustments, enzyme adaptations over an evolutionary time scale appear more relevant to cold activity than means for short-term acclimation. The strategies for increased flexibility of an enzyme leading to cold activity are numerous but not uniform. In some cases, increased flexibility is linked to a shift in primary structure (amino acid composition) of the entire protein, while in others only direct adjustments to the catalytic site of the three-dimensional macromolecule are involved. For some enzymes, including those released from the cell to perform hydrolytic functions in the environment, adjustments to flexibility are detected in the regions of the protein exposed to the solvent. Keeping an exterior shape firm as temperature drops can translate to keeping a more protected catalytic site flexible.

Considering the interface between the exterior shape of an enzyme and the solvent raise the need to consider enzyme interactions with other components in the environment, independently of the cell. For example, the extracellular polysaccharides released by *C. psychrerythraea* 34H have been observed to stabilize an extracellular protease (that it also produces) against thermal denaturation. The effective work of extracellular enzymes in the cold, for example, in hydrolyzing organic substrates to a size that can be transported into the cell, may be an important trait of heterotrophic psychrophiles. Over half of the enzymes assigned to the degradation of proteins and peptides in the *C. psychrerythraea* genome are predicted to be localized external to the cytoplasm, among the highest percentage in any completed genome (from all thermal classes). The successful infection (and reproduction) of a virus in this same psychrophile at very cold temperatures (–10 to −13 °C) suggests that at least some viruses in cold environments may carry highly cold-active enzymes for penetrating the cell membrane (**Figures 3** and **4**).

In spite of multiple strategies to achieve enough flexibility for catalytic activity in the cold, intracellularly or extracellularly, some common trends have emerged from enzyme studies regarding specific chemical modifications required to reduce the strength or number of otherwise stabilizing factors for a protein. These include reducing ion pairs, hydrogen bonds and hydrophobic interactions, inter-subunit interactions, cofactor binding, and proline and arginine content. Increasing exposure of apolar residues to the solvent, accessibility to the active site, and the clustering of glycine residues also pertain. Such trends provide means to search both available and future genomes for signs of cold adaptation. They also provide blueprints to identify or engineer proteins for applied uses in the cold, many of which have been identified by the food, detergent, and biotechnology industries, by those seeking means to remediate the contamination of cold environments, and by start-up companies interested in the possible production of cost-effective alternatives to fossil fuels that take advantage of enzymatic hydrolysis in the cold.

Cold-Shock Proteins

For the cold-adapted microbe, all nucleic acids and proteins involved in maintaining (if not synthesizing), transcribing, and translating genetic information intracellularly must be able to function in the cold. In some cases, this feat is thought to be accomplished not by primary alteration of the macromolecule itself, as already described, but by production of specific proteins that bind to them, presumably enabling proper conformation and flexibility, including required periods of destabilization. The production of cold-shock proteins to serve a similar function when mesophiles are subjected to a temperature downshift is well studied, but less is known about related responses of cold-adapted microbes to downshifts in temperature or to continuous life in the cold. Available information on psychrotolerant bacteria indicates that large numbers of cold-shock proteins (related to those in mesophiles) are always present and that production of cold-acclimation proteins is continuous in the cold, as is the expression of housekeeping genes (for basic cellular functions). By contrast, the mesophile *Escherichia coli* carries few cold-shock proteins prior to cold shock, but a temperature downshift immediately results in repression of critical housekeeping genes and induction of cold-shock (but not cold-acclimation) proteins, which is transient.

The continuous production of a variety of binding proteins to maintain proper conformation, flexibility, and function of major macromolecules thus appears to be an important trait of cold adaptation (**Figure 4**). Although work with live psychrophiles is needed, genomic sequence data support this idea; for example, the genome of *C. psychrerythraea* 34H encodes for multiple common cold-shock proteins. Furthermore, the genetic acquisition of cold-shock proteins may not require the longer term evolutionary process of vertical inheritance but may be facilitated by horizontal gene transfer. Genes for cold-shock proteins known only from the domain of Bacteria have been observed upon genomic sequencing of an uncultured population of marine Crenarchaeota from cold Antarctic waters.

Cryoprotectants and Exopolymers

In very cold environments, the cellular membranes of resident microbes are subject not only to rigidity but also to physical damage from ice-crystal formation during the freezing process. Some cold-adapted microbes are known to produce and release specific proteins that help to control the formation of ice crystals (**Figure 4**), including ice-nucleating proteins (that provide a template for crystal formation away from the cell) and antifreeze proteins that inhibit ice nucleation by dropping the freezing point or repressing the recrystallization of ice. The rate of freezing experienced by the cell also influences the degree of damage, with faster rates limiting the damage. Natural ice formations on Earth freeze slowly (producing ice crystals) relative to the vitrification process, whereby the liquid phase converts directly to solid without ice-crystal formation. When microbial cultures are vitrified in the laboratory (using liquid nitrogen at −196 °C), their cell membranes remain intact with no morphological sign of damage. When vitrified in the presence of sugars, the likelihood of recovering them in culture after thawing increases.

Small molecular weight sugars (like glycerol) have long been used as cryoprotectants in the deep-freeze (−80 °C) storage of microbes, presumably providing a buffer between cells and ice crystals. Newly discovered, however, is the overproduction of complex extracellular polysaccharides by cold-adapted bacteria, both psychrophilic and psychrotolerant, when subjected to increasingly cold temperatures, especially below the freezing point. Sea ice through its seasonal lifetime is also recently known to harbor high concentrations of sugar-based exopolymers in its liquid brine inclusions (**Figure 3**). These exopolymers, produced copiously not only by sea-ice algae but also by ice-encased bacteria, are understood to serve as natural cryoprotectants not only against potential ice-crystal damage but also by further depressing the freezing point such that more liquid water remains available within the ice matrix. In this regard, cellular coatings of exopolymers (**Figure 4**), or exopolymers available in the environment, are believed to provide a hydrated shell that helps to buffer the cell against the osmotic stress of high salt concentrations in winter sea-ice brines (**Figure 3**). Along with the possible stabilization of extracellular enzymes, this myriad of functions makes exopolymers a cold-adaptive trait worth examining in more detail. The organization of genes for exopolymers on the genome of the psychrophilic bacterium *Psychroflexus torques*, for example, suggests that they may be the result of a series of lateral gene transfer events.

Conclusion: A Model Habitat for Cold Adaptation

Considering that the ocean represents the bulk of Earth's cold biosphere, today and in the past, the annual freezing of its surface waters in polar regions takes on special significance as an important planetary driver of cold adaptation. Astronomical numbers of bacteria (10^5 in a single milliliter of seawater) pass through this frozen gauntlet annually, and over extended periods in geological time. Microbes that experience a winter in sea ice are subjected to the linked stressors of increasingly cold temperature and high brine salinity as shrinking pore space further concentrates all impurities in the source seawater, including microbes (**Figure 2**). This concentrating factor brings microbes into close proximity to each other, as verified by microscopic observations of DNA-stained cells in unmelted ice, in a liquid environment of abundant low- and high-molecular-weight organic compounds, including complex exopolymers that serve many positive functions for the trapped cells. Model calculations and observed concentrations in melted sea ice indicate that agents of lateral gene transfer (free DNA and viruses) also surround the encased cells (**Figure 2**). The virus–bacteria contact rate in a winter sea-ice brine may be as much as 600 times higher than in underlying seawater. Active virally mediated gene transfer has not yet been demonstrated, but the sea-ice environment would appear to favor it.

Even if horizontal gene transfer is not operative in sea ice, the vertical inheritance of genes for cold adaptation must be a regular occurrence. The habitat of *P. ingrahamii*, one of the most extremely psychrophilic bacteria on record (shown to reproduce at −12 °C), is sea ice. Isolates of *C. psychrerythraea* (strain 34H also grows at −12 °C) are readily cultured from sea ice. Virtually all of the canonical molecular traits of cold adaptation, along with some new ones, have been documented in the test tube or by genome analyses of such sea-ice psychrophiles. Rather than a stably cold environment, the key to the evolution of cold adaptation may be repeated exposure to the extreme cold and brine of sea ice, selecting for robustness in the face of multiple insults, including future ones like hydrostatic pressure. That salt-heavy water masses form in polar oceans actively growing sea ice and then sink to fill the cold deep ocean over time points to the concept of a cold refuge for cold-adapted bacteria during interglacial times when ice as an evolutionary driver was nonexistent, as we may witness again in this century.

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Extremophiles: Hot Environments

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Glossary

archaea A third superkingdom of life along with bacteria and eukarya.

chaperone A protein used to prevent aggregation of denatured proteins and facilitate their refolding.

ether lipid Lipids unique to archaea where isoprenoid alcohol chains are ether linked to a glycerol backbone.

histone A DNA-binding protein that compacts DNA into a nucleosome-like structure and adds thermostability to hyperthermophile DNA.

hyperthermophile An organism with an optimal growth temperature above 80 °C, primarily archaea.

methanogens Archaea that produce methane as a metabolite.

respiration Electron transfer through the cytoplasmic membrane for the purpose of energy generation and oxidative phosphorylation.

reverse gyrase A topoisomerase used to positively supercoil DNA and aid in DNA repair.

tetraether lipids Two archaeal ether lipids connected end to end by their hydrocarbon tails that form a membrane monolayer.

thermophile An organism with an optimal growth temperature between 55 and 80 °C, includes bacteria and archaea but not eukarya.

Abbreviations

APS	adenosine phosphosulfate
cDPG	cyclic 2,3-diphosphoglycerate
CoA	coenzyme A
DIP	di-*myo*-inositol-1,1′-phosphate
MGD	molybdopterin guanine dinucleotide
PRPP	5-phospho-D-ribose-1-pyrophosphate
RubisCO	ribulose-1,5-bisphosphate carboxylase/ oxygenase

Defining Statement

This article examines the microbial ecology of thermophiles and hyperthermophiles by describing factors used for DNA, protein, and cell wall thermostability; CO_2 and acetate assimilation; heterotrophy; and respiration. It also examines the evidence for the upper temperature limit for life based on pure cultures, studies on natural assemblages, and field studies.

Introduction

Life at high temperatures is classified as being either thermophilic or hyperthermophilic. Thermophiles are those organisms with optimal growth temperatures between 55 and 80 °C, whereas hyperthermophiles grow optimally at and above 80 °C. The lower bound for thermophily is based on the rarity of temperatures above this point in nature and because it is very uncommon for eukarya to grow above 55 °C. The hyperthermophile boundary is arbitrary but does largely distinguish those organisms that grow at the highest temperatures. It also has some molecular basis as generally only hyperthermophiles possess the enzyme reverse gyrase, which helps to stabilize double-stranded DNA at high temperatures. The highest known optimal growth temperatures are 105–106 °C where the slight addition of hydrostatic pressure prevents boiling.

High-temperature organisms are of interest for the information they provide on early life history, life in geothermal environments, and biochemistry. It is widely believed that the earliest life on Earth or the last common ancestor of life was either thermophilic or hyperthermophilic. Therefore, the study of modern-day (hyper)thermophiles may provide insight into the biochemical processes that occurred more than 3 billion years ago. Geothermal environments are ubiquitous on land and on the ocean floor and likely extend into vast regions of the Earth's crust. Thermophilic and hyperthermophilic microorganisms likely alter at some level the chemistry and fluid flow within these environments. High-temperature microorganisms are also useful for several industrial applications, including *in vitro* DNA replication (i.e., the polymerase chain reaction (PCR)), gas and oil recovery, laundry detergents, sweetener production, and biofuels.

Relating the biochemical characteristics of an organism to its ecology is best called physiological ecology and is the goal of this article. After an introduction to the kinds of thermophiles and hyperthermophiles found in geothermal environments, this article will examine those physiological attributes that permit these organisms to survive at high temperatures and place the information into an ecological context. This will include discussions on molecular stability at high temperatures, the upper temperature limit of life, and metabolic traits and capabilities that are relevant to biogeochemistry and microbial ecology.

Discovery: Life at High Temperatures

The presence of living organisms in geothermal hot springs was noted in *Naturalis Historia* by Pliny the Elder (23–79 AD). The first significant studies began in the nineteenth century, and early researchers recognized their importance on topics such as the structure and organization of life. Early challenges included demonstrating that the organisms were indeed alive in the springs and that the temperature was measured precisely at the organism's location because temperatures can vary significantly over a few centimeters. The first likely report of life above 80 °C came from the observation of high concentrations of microorganisms (or 'chlorophylless filamentous Schizomycetes') in an 89 °C hot spring in Yellowstone National Park sampled in 1898.

In 1965, Thomas D. Brock began detailed studies of life in the hot springs of Yellowstone National Park and elsewhere and launched the study of thermophilic microbiology. He found microorganisms were thriving in geothermal pools and streams, in some cases up to the boiling point of the fluid, and cultured the first isolates for study. After 1980, many novel hyperthermophilic genera were cultured and characterized by Karl O. Stetter, Wolfram Zillig, and others from the solfataras of Iceland and shallow marine vents along the coasts of Iceland and Italy. At the same time, microorganisms associated with deep-sea hydrothermal vents were found that utilize the steep temperature and chemical gradients to support growth. More recently, hyperthermophiles have been found in other hot environments, such as petroleum reservoirs, subsurface geothermal pools, and deep mines.

Taxonomy

Thermo(acido)philes

Although thermophiles are limited to bacteria and archaea, they are found in a wide range of environments, including coal refuse, hot-water tanks, and compost piles. This article will focus only on thermophiles from geothermal environments and are divided into the following groups. Photosynthetic bacteria generally grow up to 70–75 °C and include many cyanobacteria as well as green- and purple-sulfur bacteria such as *Chloroflexus* and *Chromatium*. Spore-forming thermophiles include *Bacillus*, *Clostridium*, and *Moorella* species. Several thermophilic Actinomycetes have been described as well as thermophilic sulfur-oxidizers (e.g., *Thiobacillus*), sulfate reducers (e.g., *Desulfovibrio*), and Gram-negative aerobes (e.g., *Thermus*). Among archaea, thermophiles are often also acidophiles. *Sulfolobus*, *Acidianus*, and *Metallosphaera* are found in acidic terrestrial hot springs and have pH optima around pH 2. *Aciduliprofundum boonei* is a marine thermoacidophile recently found in deep-sea hydrothermal vents that grows optimally at pH 4.5, which highlights the potential importance of acidophily in marine environments as well. Methanogens are found in the thermophilic temperature range as well as the hyperthermophilic range.

Hyperthermophiles

Hyperthermophily is found primarily within the archaea, which contain 33 of the 37 reported genera (**Table 1**). The remaining four genera are bacteria. Archaea are further divided into two major phyla: the Crenarchaeota and the Euryarchaeota. Hyperthermophiles are found in both, but most genera are Crenarchaeota. A third archaeal phylum is Nanoarchaeota whose sole representative is a hyperthermophilic epibiont of certain *Ignicoccus* species. There is molecular evidence for a fourth archaeal phylum, the Korarchaeota, which is believed to contain either thermophilic or hyperthermophilic members but are yet to be cultured.

In the Crenarchaeota, the Sulfolobaceae contain primarily thermoacidophiles that are found in terrestrial hot springs and grow optimally around pH 2. The Thermoproteaceae are also largely freshwater organisms but grow in more neutral environments. The Desulfurococcaceae and Pyrodictiaceae are marine hyperthermophiles that grow in mildly acidic to neutral pHs. In the Euryarchaeota, the Thermococcaceae are marine heterotrophs, whereas the Archaeoglobaceae are marine heterotrophs and autotrophs. The Methanopyraceae, Methanocaldococcaceae, and Methanothermaceae contain freshwater and marine methanogens. Hyperthermophilic bacteria are found in the Aquificaceae, Thermotogaceae, and Thermodesulfobacteriaceae. The degree of homology between the

Table 1 Taxonomy of hyperthermophiles at the superkingdom, phylum, family, and genus levels and their growth characteristics. Optimum (T_{opt}) and maximum (T_{max}) growth temperatures are the maximum known temperatures for each genus

Taxonomic group	*Metabolism*[a]	*Electron acceptors*	T_{opt} (T_{max}) (°C)
Archaea			
Crenarchaeota			
Desulfurococcaceae			
Acidolobus	H	H^+, S°	85 (92)
Aeropyrum	H	O_2	95 (100)
Caldococcus	H	S°	92 (96)
Desulfurococcus	H	H^+, S°	85 (95)
Ignicoccus	C	S°	90 (98)
Ignisphaera	H		95 (98)
Staphylothermus	H	S°	92 (98)
Stetteria	C	S°, $S_2O_3{}^{2-}$	95 (102)
Sulfophobococcus	H	H^+	85 (95)
Thermodiscus	H	H^+, S°	90 (98)
Thermosphaera	H	H^+	85 (90)
Pyrodictiaceae			
Hyperthermus	H	S°	101 (108)
Pyrodictium	C, H	FeO, S°, $S_2O_3{}^{2-}$, $SO_3{}^{2-}$	105 (110)
Pyrolobus	C	FeO, $NO_3{}^-$, O_2, $S_2O_3{}^{2-}$	106 (113)
Sulfolobaceae			
Acidianus	C	S°, O_2	88 (95)
Stygiolobus	C	S°	80 (89)
Sulfolobus	C	O_2	81 (90)
Sulfurisphaera	H	O_2, S°	84 (92)
Thermoproteaceae			
Caldivirga	H	O_2, S°, $S_2O_3{}^{2-}$, $SO_4{}^{2-}$	85 (92)
Pyrobaculum	H, FA	Fe^{3+}, FeO, $NO_3{}^-$, S°, $S_2O_3{}^{2-}$	100 (103)
Thermophilum	H	S°	88 (95)
Thermoproteus	H, FA	S°	88 (97)
Vulcanisaeta	H	Fe^{3+}, S°, $S_2O_3{}^{2-}$, $SO_4{}^{2-}$	90 (99)
Euryarchaeota			
Archaeoglobaceae			
Archaeoglobus	C, FA	$SO_4{}^{2-}$	83 (95)
Ferroglobus	C	FeO, $NO_3{}^-$, $S_2O_3{}^{2-}$	85 (95)
Geoglobus	FA	FeO	88 (90)
Methanocaldococcaceae			
Methanocaldococcus	C	CO_2	85 (91)
Methanopyraceae			
Methanopyrus	C	CO_2	98 (110)
Methanothermaceae			
Methanothermus	C	CO_2	88 (97)
Thermococcaceae			
Paleococcus	H	Fe_2S_3, O_2, S°	83 (88)
Pyrococcus	H	H^+, S°	100 (105)
Thermococcus	H	FeO, Fe_2S_3, H^+, S°	87 (93)
Nanoarchaeota			
Nanoarchaeum	H	S°	90 (98)
Bacteria			
Aquificaceae			
Aquifex	C	$NO_3{}^-$, O_2	85 (95)
Thermocrinis	C	O_2	80 (89)
Thermotogaceae			
Thermotoga	H	FeO, H^+, S°	80 (90)
Thermodesulfobacteriaceae			
Geothermobacterium	C	FeO	90 (100)

[a] C, chemolithoautotrophic; H, heterotrophic; FA, facultative autotroph.

nucleotide sequences of small-subunit ribosomal RNA from all life suggests that both bacterial and archaeal hyperthermophiles are positioned largely near the root of the phylogenetic tree. This supports the idea that phylogenetically they are the closest extant organisms to early life on the planet.

Mechanisms of Thermostability

DNA

DNA denatures *in vitro* from a double strand to two single strands (i.e., melts) at temperatures between 70 and 90 °C. The stability of the DNA secondary structure is a function of the degree of guanosine–cytosine base pairing and salt concentration, with DNA melt temperature increasing with GC content and salt. As a result, thermophiles and especially hyperthermophiles must have *in vivo* mechanisms that prevent this melting. This protection comes in the forms of protein–DNA interaction and solutes. Above 90 °C, DNA damage (e.g., depurination) occurs at rates that are 1000-fold higher than at 25 °C. Thus hyperthermophiles also require a highly efficient DNA repair mechanism.

DNA from the hyperthermophile *Pyrococcus furiosus* was fragmented into pieces that were less than 500 kb when cultures were irradiated with 2.5 kGy of γ-radiation. After irradiation and incubation for 4 h at 95 °C, its genome was fully restored and growth recommenced, thus demonstrating a highly efficient DNA repair mechanism within this organism. DNA thermostability and repair has focused primarily on a protein known as reverse gyrase, which is a type IA topoisomerase with a helicase domain. Reverse gyrase has the unique ability to introduce positive supercoiling into DNA molecules, which counters the effect of DNA melting at higher temperatures and stands in contrast to the negative supercoiling of DNA found in mesophiles. It is the only protein known to be specific to hyperthermophiles in both archaea and bacteria. Reverse gyrase is also recruited to DNA after the induction of DNA damage, showing that it is part of a broader DNA repair mechanism. It recognizes nicked DNA, recruits a protein coat to the site of damage through cooperative binding, and reduces the rate of double-stranded DNA breakage. Thus it maintains damaged DNA in a conformation that is amenable to repair. However, reverse gyrase is not a prerequisite for hyperthermophilic life. *Thermococcus kodakaraensis* with a disrupted reverse gyrase gene had retarded growth, especially at higher temperatures, but the disruption did not lead to a lethal phenotype. The gene for a RecA-like protein known as RadA and several other putative genes for DNA repair were induced in *P. furiosus* after γ irradiation, suggesting that hyperthermophiles possess a spectrum of DNA repair mechanisms. Hyperthermophiles also possess up to ten copies of their genomes, which may readily facilitate DNA repair by homologous recombination.

The thermostability of the DNA double helix in hyperthermophilic archaea is also due to DNA interaction with double-stranded DNA-binding proteins. Many Euryarchaeota produce histone-like proteins that show sequence homology to the core fold region of eukaryotic histones but are shorter than eukaryotic histones. These proteins form dimers (or monomeric pseudodimers with twofolds) that must assemble into tetramers to bind DNA. The tetrameric form has been shown to compact DNA both *in vitro* and *in vivo* and adds to the thermostability of the double-stranded DNA. Crenarchaeota lack histone-like proteins but have other DNA-binding proteins that also compact DNA and increase the melting temperature of double-stranded DNA *in vitro*. These archaeal DNA-binding proteins are more likely for DNA packaging and nucleosome formation than for DNA stability.

The secondary structure of nucleic acids is also stabilized by the accumulation of specific organic osmolytes. The melting temperature of DNA from *Methanothermus fervidus* increased with the concentration of cyclic 2,3-diphosphoglycerate (cDPG), a compatible solute that is produced by thermophilic and hyperthermophilic methanogens. Another osmolyte unique to hyperthermophiles, di-*myo*-inositol-1,1′-phosphate (DIP), may also serve as a DNA thermoprotectant as this compound increases in concentration with organism growth temperature (see 'Protein'). Polycationic polyamines are also observed in hyperthermophiles and increase the melting temperature of DNA. Concentrations of up to 0.4 g% (d.w. cell biomass) of putrescine, spermidine, norspermine, thermospermine, and spermine were detected in various *Sulfolobus* strains. Norspermine and norspermidine occur only in hyperthermophilic archaea, and these organisms typically contain a greater diversity of polyamines than other organisms.

Protein

A remarkable feature of thermophiles and hyperthermophiles is the broad thermostability of proteins across all functional classes and forms. Perhaps the most significant finding is that enzymes from hyperthermophiles, thermophiles, and mesophiles have no pattern of systematic structural differences that provides thermostability. There are small differences within a related group of proteins with varying optimal temperatures, but the changes will be different for a different related group of proteins. Furthermore, only a few amino acid substitutions are necessary to bring about significant changes in protein thermal stability.

There is strong evidence in general terms for an inverse correlation between protein thermal stability and molecular flexibility (i.e., a less flexible protein will be more thermostable). A balance must be struck between stabilizing and destabilizing interactions to meet the conflicting demands of thermostability and catalytic function, respectively. The structural basis of thermostability in hyperthermophilic proteins varies for different proteins with some commonality between certain examples. A comparison of crystal structures of hyperthermophilic, thermophilic, and mesophilic orthologous proteins shows that some of the more common structural changes found in thermophilic proteins are as follows: (1) an increase in the number of ion pairs, (2) an increase in the number of hydrogen bonds between positively charged side chains and neutral oxygens, (3) more extensive α helix secondary structure, (4) a decrease in the number of internal cavities, (5) a decrease in surface-to-volume ratios by shortening surface loops, (6) an increase in the number of hydrophobic residues as the hydrophobic effect increases with temperature, and (7) oligomerization.

In addition to these intrinsic factors, there are numerous extrinsic factors that likewise enhance protein stability at high temperatures. These include a chaperone protein (thermosome) that is unique to hyperthermophilic archaea, is highly abundant at all hyperthermophile growth temperatures, and is the primary protein produced during heat shock. The chaperone from *Sulfolobus solfataricus* prevents the aggregation of denatured target protein, catalyzes the refolding of the protein upon the addition of K^+, and releases the substrate after ATP hydrolysis. Hyperthermophiles also produce nonproteinaceous osmolytes that serve to stabilize proteins at high temperatures. Thermophilic and hyperthermophilic methanogens produce cDPG, which stabilizes thermolabile proteins *in vitro* at superoptimal temperatures. Other hyperthermophiles produce DIP. Like cDPG, DIP stabilized proteins *in vitro* at superoptimal temperatures. The concentration of DIP in *P. furiosus* increases 20-fold during heat shock, further suggesting that DIP functions specifically to stabilize macromolecules at high temperatures. It was also shown that elevated pressure stabilizes numerous hyperthermophilic enzymes, whereas their mesophilic counterparts were unaffected or inhibited by elevated pressure. Elevated pressure is believed to cause increased packing of the molecule, which likewise imparts increased structural rigidity and thermostability to these proteins.

Cell Wall

The maintenance of membrane fluidity is essential for normal cell function, and the mechanisms for maintaining stable and adaptive membranes in hyperthermophilic archaea and bacteria differ significantly from each other. Studies on artificial membranes (i.e., liposomes) demonstrate that the membranes of hyperthermophiles have evolved mechanisms for maintaining a liquid crystal state at high temperatures. In archaea, lipids are composed of isoprenoid alcohol chains that are ether-linked to the glycerol backbone. Ether linkages are also found in low proportions in some thermophilic bacteria and may mark a thermophilic adaptation. The lipid bilayer is also cross-linked at certain points by C_{40} trans-membrane phytanyl chains (i.e., tetraether lipids). Membranes that contain these membrane-spanning lipids are much more thermostable than those formed from phosphodiester lipids. The proportion of tetraether-to-diether lipids in *Methanocaldococcus jannaschii* increased significantly with temperature, supporting the idea that an increased proportion of tetraether lipids in archaeal membranes contributes to cell wall thermostability.

Upper Temperature Limit of Life

Hyperthermophile Culture Studies

Hyperthermophiles have the highest known growth temperatures for life, and most of what is known about them has come through pure culture studies under well-defined and regulated conditions in the laboratory. From these studies, the highest optimal growth temperature for an organism is 105–106 °C (**Table 1**). The heterotrophic archaea *Hyperthermus butylicus* and *Pyrodictium abyssi* have maximum growth temperatures of 108 and 110 °C, respectively. They grow on peptides and their growth is stimulated by the addition of H_2, CO_2, and S°. The obligately chemolithoautotrophic archaea *Pyrodictium occultum* and *Pyrodictium brockii* grow up to 110 °C; require H_2 and S°, $S_2O_3^{2-}$, or SO_3^{2-} for growth; and are stimulated by the addition of yeast extract (i.e., mixed organic compounds). After prolonged incubation (9 days at 96 °C), very few *Pyrodictium* cells are present singly in suspension. Rather, the cells are connected by ultrathin fibers that form a network. This may be a mechanism to enhance the thermostability of these cells. The methanogenic archaeon *Methanopyrus kandleri* also grows at temperatures up to 110 °C and requires H_2 and CO_2 to produce CH_4. The cultured organisms with the highest growth temperatures are the obligately chemolithoautotrophic archaea *Pyrolobus fumarii* and Pyrodictiaceae strain 121, which grow up to 113 and 121 °C, respectively. *P. fumarii* requires H_2 as an electron donor and can use NO_3^-, $S_2O_3^{2-}$, iron oxide hydroxide, and low levels of O_2 as electron acceptors. In contrast, strain 121 can use either H_2 or formate as an electron donor but can only use iron oxide hydroxide as an electron acceptor.

A parameter related to the upper temperature limit of life is pressure. Any life above 100 °C requires pressure >0.1 MPa to maintain a liquid environment. Most marine hyperthermophiles are present in deep-sea hydrothermal vent sites where the *in situ* pressure is 20–45 MPa (pressure increases 0.1 MPa, or 1 times atmospheric pressure, for every 10 m of water depth). Pressure effects on hyperthermophiles are generally favorable for growth at high temperatures. Relative to low pressures (0.1–3 MPa), the maximum growth temperature increases 2–6 °C for *Pyrococcus*, *Thermococcus*, and *Desulfurococcus* species when incubated at *in situ* pressures. For other hyperthermophiles, although their optimum growth temperature does not increase with pressure, their rate of growth does increase significantly at elevated pressure. For *M. jannaschii*, hyperbaric pressure significantly increases its growth rate at 86 °C but does not increase its optimum growth temperature. However, the maximum temperature for CH_4 formation increases from 92 °C at 0.8 MPa to 98 °C at 25 MPa. Likewise, methanogenesis rates are higher at higher pressures.

Laboratory Studies on Natural Microbial Assemblages

Natural assemblages of microorganisms collected from deep-sea hydrothermal vent sites have also been studied under controlled conditions in the laboratory. Petroleum-rich sediment cores from Guaymas Basin showed maximum sulfate reduction activities at 90 °C with additional activity between 105 and 110 °C. The hyperthermophilic sulfate reducer *Archaeoglobus profundus* was cultured from these same sediment samples, which has a maximum growth temperature of 90 °C and most likely is responsible for the 90 °C sulfate reduction activity peak. The organisms responsible for sulfate reduction between 100 and 110 °C are unknown. A natural assemblage of microorganisms collected from high-temperature black smoker fluids was incubated in solid gel material (GELRITE) that is stable at temperatures up to 120 °C. Colonies of microorganisms formed in the gel at 115 and 120 °C at 7 and 27 MPa. The largest colonies (~0.5 mm diameter) formed at 27 MPa, which again demonstrates the positive influence of pressure on the growth of microorganisms at high temperatures. Growth also may have been enhanced by the presence of a solid matrix to which cells attach themselves.

Field Observations

Analyses for biomolecules and intact cells have been performed on exiting hydrothermal fluids, sulfide ore deposits, and sediment and rock core samples. The primary difficulty has been determining what proportion of the material originated from seawater and cooler sulfide ore material that frequently contaminates samples. An *in situ* incubator containing interior melting point standards was deployed on top of a black smoker at Guaymas Basin (Vent 1). Microbial colonization was observed where a 125 °C sensor had melted but not a neighboring 140 °C sensor, although exposure to 125 °C may have been transient. In a sulfide ore deposit from a deep-sea hydrothermal vent site, the highest concentrations of ether lipids and intact fluorescent cells were found in mineral layers consisting primarily of anhydrite and Zn-Fe sulfides, which suggests that their temperatures were between 100 and 140 °C.

Field evidence for life above 100 °C is circumstantial due to the difficulties of obtaining accurate temperature measurements on the spatial scales necessary, the temporal variations in temperature at a given site, the uncertain origin of the biomass analyzed, and the absence of direct microbial activity measurements. These results are speculative rather than conclusive, and await further detailed analyses for verification and identification of indigenous microorganisms and their metabolic traits.

Metabolism and Growth

CO_2 and Acetate Assimilation

The assimilation of CO_2 by autotrophs is often associated with the Calvin cycle. However, this pathway is generally absent in thermophilic bacteria and is completely absent in archaea. Alternative CO_2 assimilation pathways are generally used in these organisms and include the coenzyme A (CoA) pathway, the reductive citric acid cycle, the 3-hydroxypropionate cycle, and the 4-hydroxybutyrate cycle. These pathways were discovered in part by studying anaerobic thermophiles. Acetate assimilation can occur using the citramalate cycle, the glyoxylate shunt, or by reversing a portion of the acetyl-CoA pathway. Thermophilic bacteria and archaea use all of these pathways for inorganic carbon assimilation in some capacity. An archaea-specific version of the acetyl-CoA pathway is used by Euryarchaeota with CH_4 production as added steps to the pathway in methanogens (**Figure 1**). Crenarchaeota use the reductive citric acid cycle, which is lacking in most hyperthermophilic Euryarchaeota, as well as the 3-hydroxypropionate, the 4-hydroxybutyrate, and citramalate cycles. The pathway for CO_2 assimilation in the Pyrodictiaceae is largely unknown and likely represents a novel autotrophic pathway.

Acetyl-CoA pathway and methanogenesis

Moorella thermoacetica and *Moorella thermoautotrophica* (formerly *Clostridium thermoaceticum* and *C. thermoautotrophicum*) are facultative autotrophs and obligately anaerobic spore formers. When grown on glucose, these organisms produce

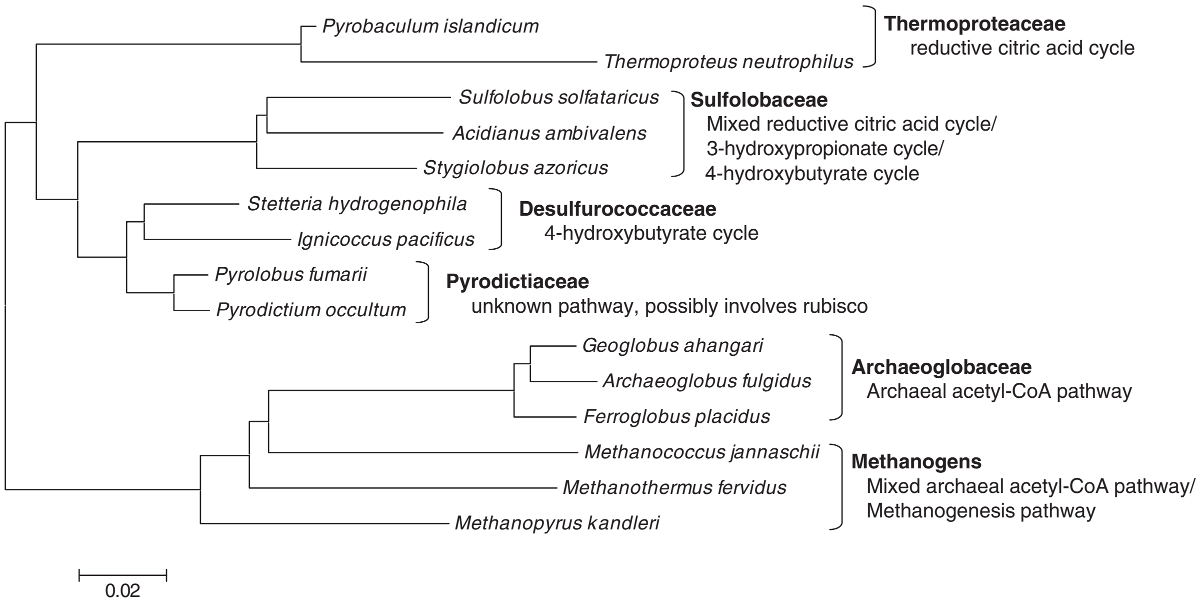

Pyrobaculum islandicum
Thermoproteus neutrophilus
Thermoproteaceae
reductive citric acid cycle
Sulfolobus solfataricus
Acidianus ambivalens
Stygiolobus azoricus
Sulfolobaceae
Mixed reductive citric acid cycle/
3-hydroxypropionate cycle/
4-hydroxybutyrate cycle
Stetteria hydrogenophila
Ignicoccus pacificus
Desulfurococcaceae
4-hydroxybutyrate cycle
Pyrolobus fumarii
Pyrodictium occultum
Pyrodictiaceae
unknown pathway, possibly involves rubisco
Geoglobus ahangari
Archaeoglobus fulgidus
Ferroglobus placidus
Archaeoglobaceae
Archaeal acetyl-CoA pathway
Methanococcus jannaschii
Methanothermus fervidus
Methanopyrus kandleri
Methanogens
Mixed archaeal acetyl-CoA pathway/
Methanogenesis pathway
0.02

Figure 1 Phylogeny of chemolithoautotrophic archaea based on 16S rRNA sequence homologies and the CO_2 assimilation pathways for each family or group of organisms.

three molecules of acetate per glucose and little CO_2. This led to the suggestion that glucose is first oxidized to two molecules each of acetate and CO_2, followed by the production of a third acetate from the two CO_2 molecules. $^{14}CO_2$ and $^{13}CO_2$ incubation experiments confirmed that both carbons of acetate are labeled during growth. Incubation with ^{13}C-formate led to the labeling of the methyl group in acetate and suggested that formate is an intermediate in the pathway. The isolation of the autotrophic acetogen *Acetobacterium woodii* confirmed CO_2 assimilation can occur by means other than by the Calvin cycle, which is now known as the acetyl-CoA pathway (or Wood–Ljungdahl pathway). In addition to these thermophilic bacteria, an archaeal version of the pathway is used by autotrophic members of the Archaeoglobaceae and by all methanogens growing on H_2 and CO_2.

The acetyl-CoA pathway reduces and condenses two molecules of CO_2 to form one molecule of acetyl-CoA (**Figure 2**). One CO_2 molecule is reduced in a series of reduction steps to a methyl group ligated to a C_1 carrier. In bacteria, the C_1 carriers are tetrahydrofolate and coenzyme E; in archaea, they are methanofuran and tetrahydromethanopterin. Another difference between the pathways in bacteria and archaea is the use of a soluble 5-deazaflavin called coenzyme F_{420} by archaea that carries two electrons but only one hydrogen. The enzymes in bacteria and archaea that catalyze these steps are conserved functionally but have little-to-no sequence homologies between them. The key enzyme in the pathway is carbon monoxide dehydrogenase/acetyl-CoA synthase, which is a highly conserved protein across superkingdoms. This enzyme reduces the second CO_2 molecule to a carbon monoxyl group, ligates it to the methyl group from the C_1 carrier, and then adds CoA to form acetyl-CoA. Methanogens use three additional enzymes (methyl-MPT:CoM methyltransferase, methyl-CoM reductase, and heterodisulfide reductase) to dispose of electrons during their anaerobic respiration using methyl-MPT as their terminal electron acceptor (**Figure 2**).

Reductive citric acid cycle

Like *M. thermoacetica* and *M. thermoautotrophica*, it was shown in the photosynthetic green sulfur bacterium *Chlorobium limicola*, the purple sulfur bacterium *Chromatium vinosum*, and the purple nonsulfur bacterium *Rhodospirillum rubrum* that CO_2 assimilation occurs by a pathway other than the Calvin cycle. The concept of the reductive citric acid cycle (**Figure 3**) had its origin in the discovery of ferredoxin-dependent reductive carboxylation reactions: pyruvate synthase and 2-oxoglutarate (formerly α-ketoglutarate) synthase. They are driven by the strong reducing potential of reduced ferredoxin and complement the irreversible NAD^+-dependent pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase reactions. Other key findings were enzymes that produce oxaloacetate and acetyl-CoA from citrate and complement the irreversible citrate synthase step in the oxidative citric acid cycle. In *C. limicola* and *Desulfobacter hydrogenophilus*, citrate

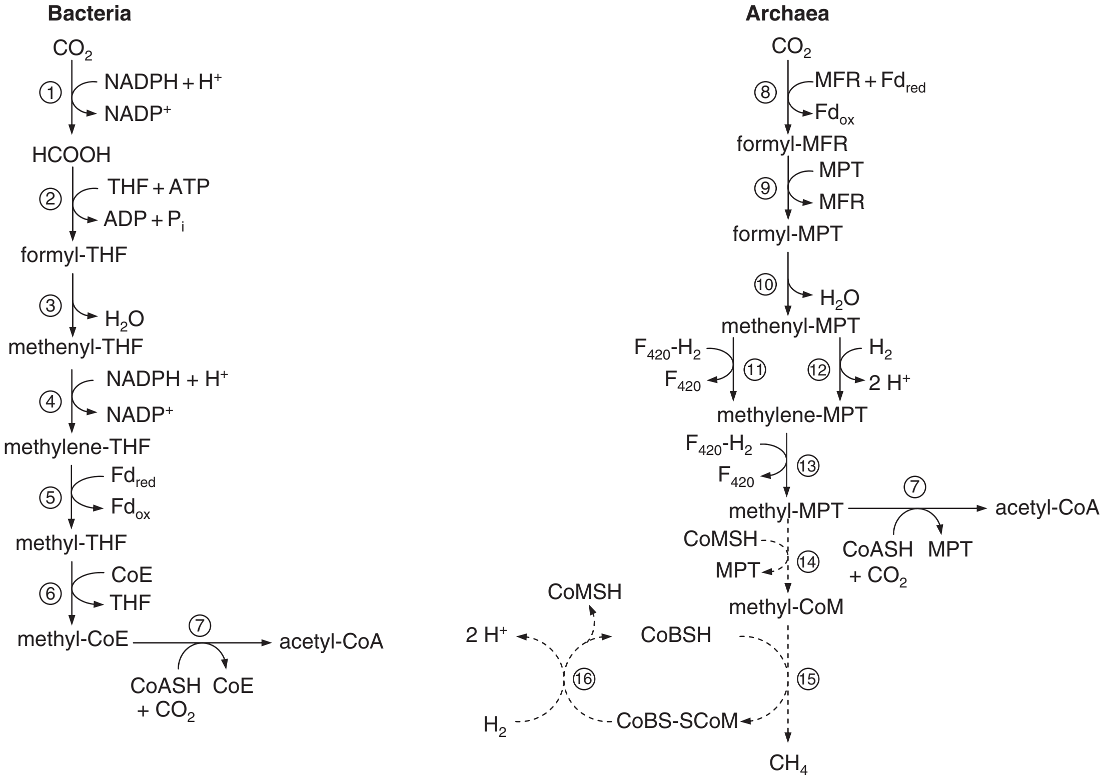

Bacteria
CO2
① NADPH + H+
NADP+
HCOOH
② THF + ATP
ADP + Pi
formyl-THF
③ H2O
methenyl-THF
④ NADPH + H+
NADP+
methylene-THF
⑤ Fdred
Fdox
methyl-THF
⑥ CoE
THF
methyl-CoE
⑦
acetyl-CoA
CoASH + CO2
CoE
Archaea
CO2
⑧ MFR + Fdred
Fdox
formyl-MFR
⑨ MPT
MFR
formyl-MPT
⑩ H2O
methenyl-MPT
F420-H2
F420
⑪
⑫ H2
2 H+
methylene-MPT
F420-H2
F420
⑬
methyl-MPT
⑦
acetyl-CoA
CoASH + CO2
MPT
CoMSH
MPT
⑭
methyl-CoM
CoMSH
2 H+
CoBSH
⑯
⑮
H2
CoBS-SCoM
CH4

Figure 2 The acetyl-CoA pathways in bacteria and archaea. The enzymes are as follows: 1, formate dehydrogenase; 2, formyl-THF synthetase; 3, methenyl-THF cyclohydrolase; 4, methylene-THF dehydrogenase; 5, methylene-THF reductase; 6, methyltransferase; 7, carbon monoxide dehydrogenase; 8, formyl-MF dehydrogenase; 9, formyl-MF:MPT formyltransferase; 10, methenyl-MPT cyclohydrolase; 11, F_{420}-dependent methylene-MPT dehydrogenase; 12, F_{420}-independent methylene-MPT dehydrogenase; 13, methylene-MPT reductase; 14, methyl-MPT:CoMSH methyltransferase; 15, methyl-CoM reductase; and 16, heterodisulfide reductase. THF, tetrahydrofolate; MF, methanofuran; MPT, tetrahydromethanopterin; CoE, coenzyme E; CoM, coenzyme M; CoB, coenzyme B; Fd, electron carrier ferredoxin; and F_{420}, electron carrier coenzyme F_{420}. The dashed lines show the enzyme steps that are unique to methanogenesis.

cleavage is accomplished in a single step by ATP citrate lyase (eqn [1]):

$$\text{citrate} + \text{ATP} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{P}_i \qquad [1]$$

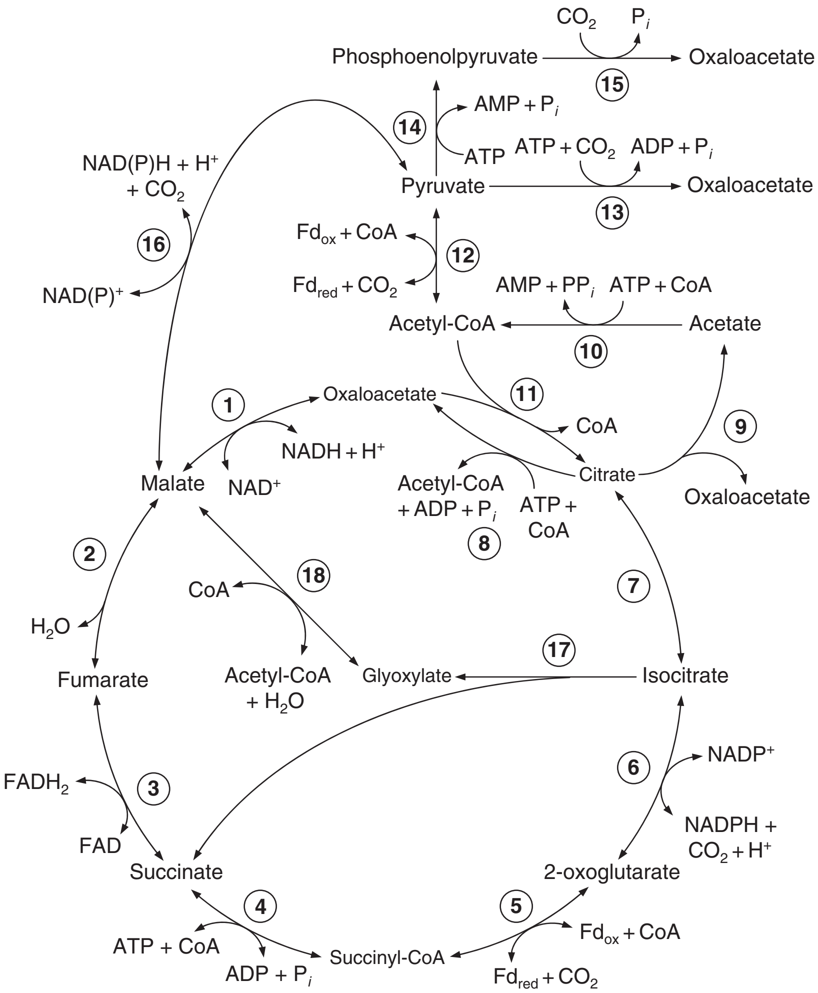
This protein is the citrate cleavage enzyme that is most commonly associated with the reductive citric acid cycle. In the thermophilic bacterium *Hydrogenobacter thermophilus* TK-6, citrate cleavage is catalyzed in two steps by citryl-CoA synthetase (eqn [2]) and citryl-CoA lyase (eqn [3]):

$$\text{citrate} + \text{ATP} + \text{CoA} \rightarrow \text{citryl-CoA} + \text{ADP} + \text{P}_i \qquad [2]$$

$$\text{citryl-CoA} \rightarrow \text{oxaloacetate} + \text{acetyl-CoA} \qquad [3]$$

The first evidence for the reductive citric acid cycle in archaea came from the study of thermoacidophile *Acidianus brierleyi* (formerly *Sulfolobus brierleyi*). Pulse labeling of autotrophically grown *A. brierleyi* with $^{14}CO_2$ showed the formation of labeled malate, citrate, aspartate, and glutamate. Initially all of the citric acid cycle enzymes and pyruvate synthase were measured in cell extracts of *A. brierleyi* grown autotrophically. However, the lack of ATP citrate lyase activity and the presence of 3-hydroxypropionate cycle activities led to the suggestion that the organism uses this latter pathway for CO_2 assimilation. It was since shown that autotrophically grown *A. brierleyi* does possess ATP citrate lyase activity but it requires covalent modification by acetylation for activity. Therefore, the organism appears to use a combination of the reductive citric acid cycle and the 3-hydroxypropionate cycle for CO_2 assimilation.

The hyperthermophilic archaeon *Thermoproteus neutrophilus* was grown autotrophically and pulse labeled with ^{14}C- and ^{13}C-succinate, yielding labeled malate, glutamate, and aspartate. This and the presence of all of the activities of the citric acid cycle enzymes, pyruvate synthase, and ATP citrate lyase suggest that it also uses the reductive citric acid cycle for CO_2 assimilation. The presence of pyruvate synthase, 2-oxoglutarate synthase, and ATP citrate lyase activities in the hyperthermophilic archaeon *Pyrobaculum islandicum* grown autotrophically suggests that this organism likewise uses this pathway.



CO_2
P_i
Phosphoenolpyruvate
Oxaloacetate
⑮
$AMP + P_i$
⑭
ATP
$ATP + CO_2$
$ADP + P_i$
$NAD(P)H + H^+$
$+ CO_2$
Pyruvate
Oxaloacetate
⑬
$Fd_{ox} + CoA$
⑯
⑫
$NAD(P)^+$
$Fd_{red} + CO_2$
$AMP + PP_i$
$ATP + CoA$
Acetyl-CoA
Acetate
⑩
①
Oxaloacetate
⑪
CoA
⑨
$NADH + H^+$
Malate
NAD^+
Acetyl-CoA
$+ ADP + P_i$
ATP +
CoA
Citrate
Oxaloacetate
⑧
②
CoA
⑱
⑦
H_2O
⑰
Fumarate
Acetyl-CoA
$+ H_2O$
Glyoxylate
Isocitrate
$NADP^+$
⑥
$FADH_2$
③
NADPH +
$CO_2 + H^+$
FAD
Succinate
2-oxoglutarate
④
⑤
ATP + CoA
$ADP + P_i$
Succinyl-CoA
$Fd_{ox} + CoA$
$Fd_{red} + CO_2$

Figure 3 The citric acid cycle, the glyoxylate shunt, and related enzymes. The enzymes are as follows: 1, malate dehydrogenase; 2, fumarase; 3, fumarate reductase/succinate dehydrogenase; 4, succinyl-CoA synthetase; 5, 2 oxoglutarate synthase; 6, isocitrate dehydrogenase; 7, aconitase; 8, either ATP citrate lyase (one step) or citryl-CoA synthase and citryl-CoA lyase (two steps); 9, citrate lyase; 10 AMP-forming acetyl-CoA synthetase; 11, citrate synthase; 12, pyruvate synthase; 13, pyruvate carboxykinase; 14, phosphoenolpyruvate synthetase; 15, phosphoenolpyruvate carboxylase; 16, malic enzyme; 17, isocitrate lyase; and 18, malate synthase. Fd, electron carrier ferredoxin. Copyright © American Society for Microbiology, *Journal of Bacteriology*, vol.188, pp. 4350–4355, 2006.

However, for both *T. neutrophilus* and *P. islandicum*, it was suggested that the ATP citrate lyase activities are too low to account for all of the CO_2 assimilated. It was subsequently shown for *P. islandicum* that acetylated citrate lyase (eqn [4]) and AMP-forming acetyl-CoA synthase (eqn [5]) activities increase significantly in cells grown autotrophically relative to those grown heterotrophically and are higher than the ATP citrate lyase activities measured.

$$\text{citrate} \rightarrow \text{oxaloacetate} + \text{acetate} \qquad [4]$$

$$\text{acetate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i \qquad [5]$$

Therefore, there appears to be a third mechanism for citrate cleavage in thermophiles that requires covalent modification by acetylation.

All 8 of the citric acid cycle enzymes are present within 20 of the 46 archaeal genome sequences currently available (**Figure 4**). The complete cycle is found in all organisms within the Thermoproteales, the Sulfolobales, and *Aeropyrum pernix* in the Crenarchaeota and in all Halobacteriales (i.e., extreme halophiles) and Thermoplasmales in the Euryarchaeota. Only a portion of the cycle is found in the Thermococcales, the Archaeoglobales, the Desulfurococcales (except *A. pernix*), and all methanogens. The distributions of the citric acid cycle enzymes in both archaeal phyla and the acetyl-CoA pathway enzymes (only in Euryarchaeota) suggest that the last common archaeal ancestor contained the complete citric acid cycle and that portions of the cycle were lost in the methanogens, Archaeoglobales, and Thermococcales, and currently it only serves to produce

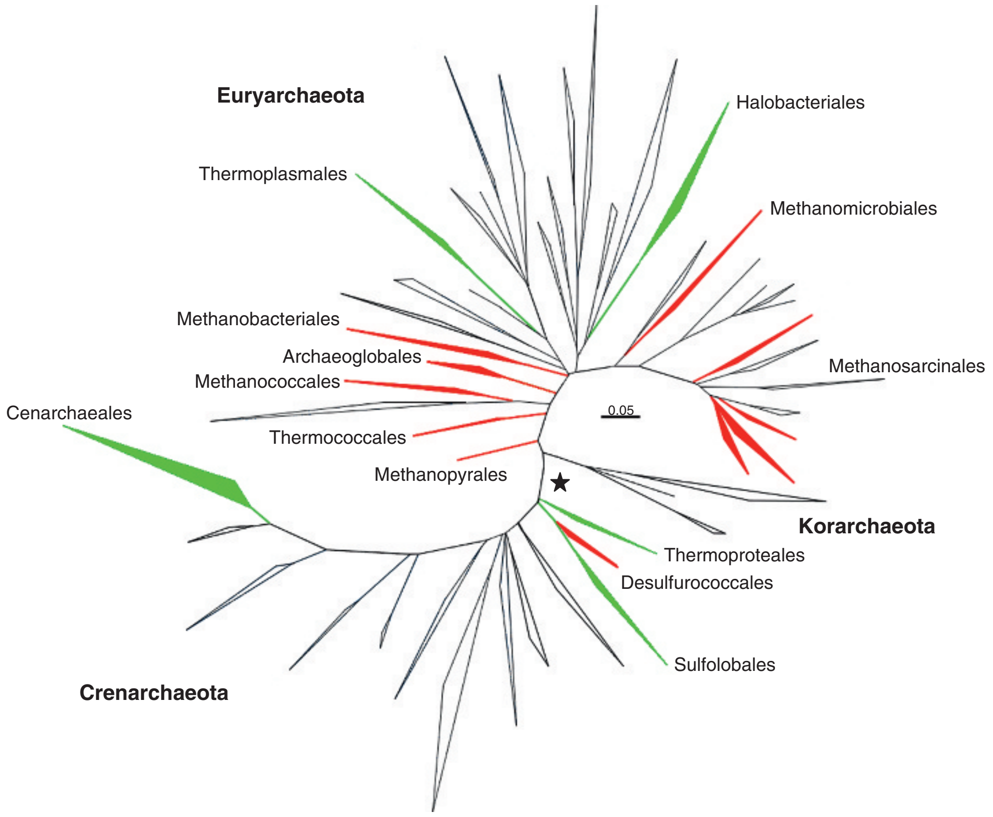


Figure 4 A 16S rRNA tree of the archaea. Those taxonomic orders that contain all of the genes that encode for citric acid cycle enzymes are shown in green, whereas those with only a subset of these genes are in red. Uncultivated orders are shown as unfilled groups. The star indicates the location of the root of the tree. The tree was constructed as described previously. The scale bar represents 0.05 changes per nucleotide. Adapted by permission from Macmillan Publishers Ltd: Schleper C, Jurgens G, and Jonuscheit M (2005) Genomic studies of uncultivated Archaea, *Nature Reviews,* vol. 3, pp. 479–488. Copyright 2005.

intermediates for some biosynthesis reactions in these organisms.

3-hydroxypropionate cycle

Chloroflexus aurantiacus is a thermophilic green nonsulfur bacterium that is a facultative photoautotroph and an anaerobe. Cultures grown photoautotrophically with H_2 and CO_2 lack ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and ribulose-5-phosphate kinase (formerly phosphoribulokinase) activities, which are the key enzymes of the Calvin cycle. They also lack ATP citrate lyase and 2-oxoglutarate synthase activities that are necessary for the reductive citric acid cycle. However, *C. aurantiacus* secretes 3-hydroxypropionate during phototrophic growth, which suggests that it is an intermediate of CO_2 assimilation. This led to the discovery of the 3-hydroxypropionate cycle where two molecules of CO_2 are assimilated to form glyoxylate (**Figure 5**). The carboxylation reactions are catalyzed at two points in the cycle by a single bifunctional enzyme called acetyl-CoA/propionyl-CoA carboxylase. Many of the enzymes in the cycle overlap with those of the citric acid cycle to form malate, which is then split to form glyoxylate and regenerate acetyl-CoA.

As mentioned previously, the lack of ATP citrate lyase activity in the thermoacidophilic archaeon *A. brierleyi* and the presence of acetyl-CoA carboxylase and propionyl-CoA carboxylase activities led to the suggestion that this organism uses the 3-hydroxypropionate cycle for CO_2 assimilation. Acetyl-CoA carboxylase and propionyl-CoA carboxylase activities were also measured in autotrophically grown cell extracts from *Sulfolobus metallicus* and *Acidianus infernus*. The subsequent discovery of ATP citrate lyase activity in *A. brierleyi* after acetylation and the activities of both the citric acid and 3-hydroxypropionate cycles suggest that this organism uses a combination of these pathways for CO_2 assimilation.

4-hydroxybutyrate cycle

Ignicoccus species are hyperthermophilic obligately autotrophic archaea that belong to the Desulfurococcaceae. *Ignicoccus pacificus* and *Ignicoccus islandicus* lack ATP citrate lyase, 2-oxoglutarate synthase, carbon monoxide dehydrogenase, and acetyl-CoA/propionyl–CoA carboxylase activities that are indicative of the reductive citric acid

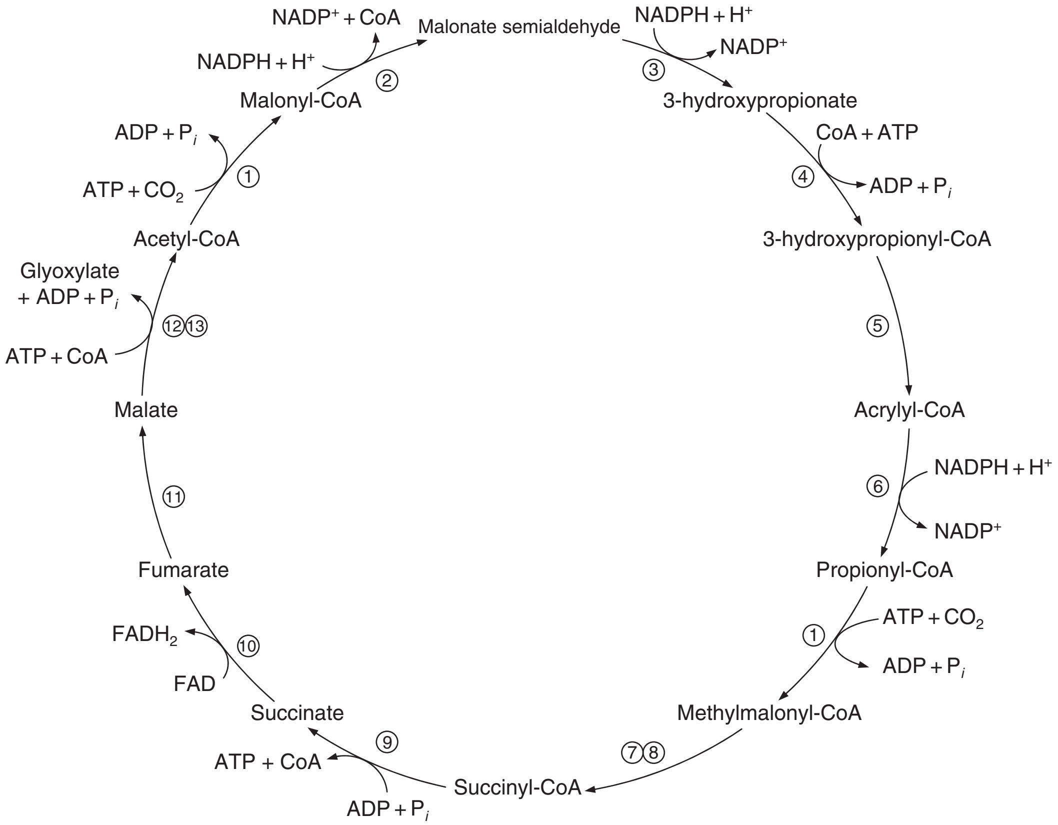

NADP⁺ + CoA
Malonate semialdehyde
NADPH + H⁺
NADP⁺
NADPH + H⁺
②
③
Malonyl-CoA
3-hydroxypropionate
ADP + P_i
CoA + ATP
①
④
ATP + CO_2
ADP + P_i
Acetyl-CoA
3-hydroxypropionyl-CoA
Glyoxylate
+ ADP + P_i
⑫⑬
⑤
ATP + CoA
Malate
Acrylyl-CoA
NADPH + H⁺
⑪
⑥
NADP⁺
Fumarate
Propionyl-CoA
ATP + CO_2
$FADH_2$
⑩
①
ADP + P_i
FAD
Succinate
Methylmalonyl-CoA
⑨
⑦⑧
ATP + CoA
Succinyl-CoA
ADP + P_i

Figure 5 The 3-hydroxypropionate cycle. The enzymes are as follows: 1, acetyl-CoA/propionyl-CoA carboxylase; 2, malonyl-CoA reductase; 3, 3-hydroxypropionate dehydrogenase; 4, 3-hydroxypropionyl-CoA hydrolase; 5, acrylyl-CoA hydratase; 6, acrylyl-CoA dehydrogenase; 7, methylmalonyl-CoA epimerase; 8, methylmalonyl-CoA mutase; 9, succinyl-CoA synthetase; 10, succinate dehydrogenase; 11, fumarase; 12, malyl-CoA synthetase; and 13, malyl-CoA lyase.

cycle, the acetyl-CoA pathway, and the 3-hydroxypropionate cycle, respectively. It was shown that *Ignicoccus hospitalis* assimilates CO_2 in two steps using pyruvate synthase and phosphoenolpyruvate carboxylase (**Figure 6**), and many of the enzymes are the same as those used in the reductive citric acid cycle. However, because the organism lacks 2-oxoglutarate synthase, it reduces succinyl-CoA in two enzymatic steps to form 4-hydroxybutyrate and eventually acetoacetyl-CoA. In the final step, acetoacetyl-CoA is cleaved to form two molecules of acetyl-CoA, one of which brings the cycle back to its starting point, yielding the net formation of one acetyl-CoA.

Interestingly, the thermoacidophilic archaeon *Metallosphaera sedula*, which is a close relative of *Sulfolobus* and *Acidianus* species, uses a mixture of the 3-hydroxypropionate and 4-hydroxybutyrate cycles. CO_2 is assimilated using acetyl-CoA/propionyl-CoA carboxylase, as is found in the 3-hydroxypropionate cycle. However, instead of forming glyoxylate and acetyl-CoA from succinyl-CoA as is found in the 3-hydroxypropionate cycle, *M. sedula* converts succinyl-CoA into two molecules of acetyl-CoA via 4-hydroxybutyrate using the same enzymes found in the 4-hydroxybutyrate cycle. Therefore, across all of the autotrophic Crenarchaeota (with the possible exception of the Pyrodictiaceae), CO_2 assimilation often involves a mixture of the reductive citric acid cycle, the 3-hydroxypropionate cycle, and the 4-hydroxybutyrate cycle, and many of the same enzymes (i.e., malate dehydrogenase, fumarase, succinate dehydrogenase/fumarate reductase, succinyl-CoA synthetase, pyruvate synthase, PEP synthetase, and PEP carboxylase) are used, suggesting that there is an evolutionary relationship between these CO_2 assimilation pathways.

Other possible CO_2 assimilation pathways

Hyperthermophilic archaea belonging to the Pyrodictiaceae may possess novel pathways for CO_2 assimilation. Autotrophically grown *P. fumarii*, *P. abyssi* and *P. occultum* grown on yeast extract with H_2 and CO_2 all had pyruvate synthase activity but lacked 2-oxoglutarate synthase activity and generally lacked other enzymes of the citric acid cycle needed for the 3-hydroxypropionate and 4-hydroxybutyrate cycles. *P. abyssi* and *P. occultum* also lack carbon monoxide dehydrogenase/acetyl-CoA synthase activity. Therefore, these organisms do not appear to use

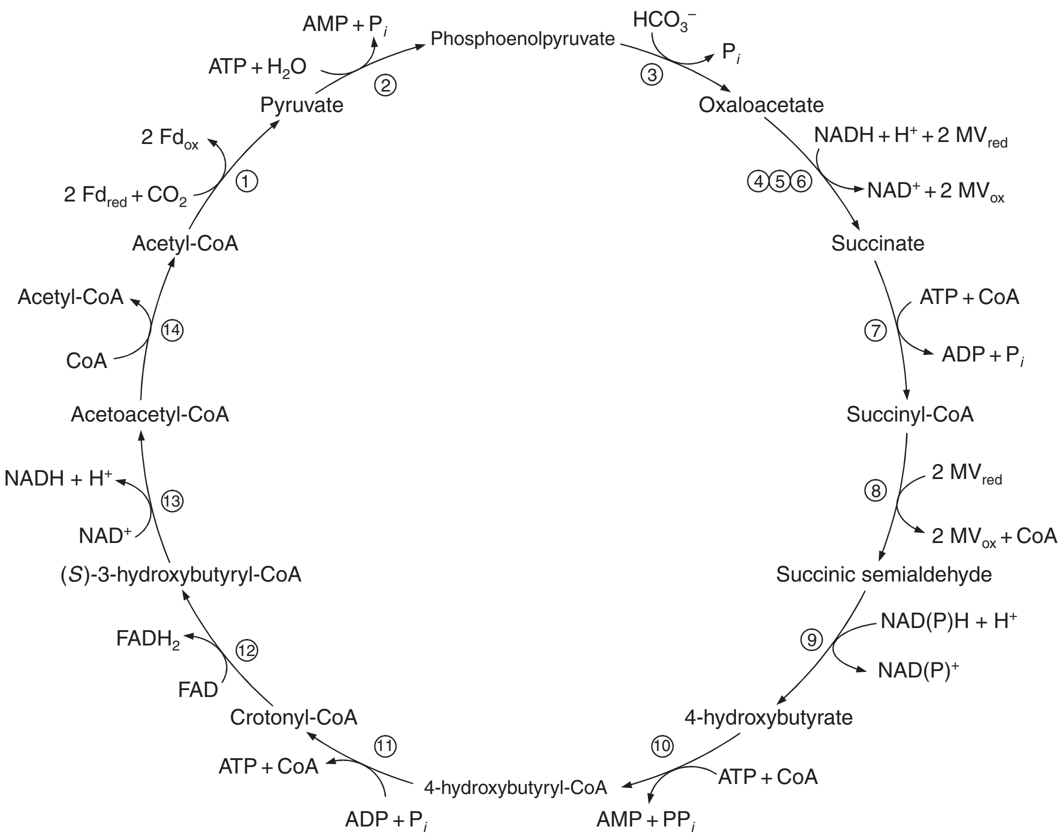

Phosphoenolpyruvate
AMP + P$_i$
ATP + H$_2$O
②
HCO$_3^-$
P$_i$
③
Oxaloacetate
Pyruvate
2 Fd$_{ox}$
2 Fd$_{red}$ + CO$_2$
①
NADH + H$^+$ + 2 MV$_{red}$
④⑤⑥
NAD$^+$ + 2 MV$_{ox}$
Succinate
Acetyl-CoA
Acetyl-CoA
⑭
CoA
ATP + CoA
⑦
ADP + P$_i$
Succinyl-CoA
Acetoacetyl-CoA
NADH + H$^+$
⑬
NAD$^+$
2 MV$_{red}$
⑧
2 MV$_{ox}$ + CoA
(S)-3-hydroxybutyryl-CoA
Succinic semialdehyde
FADH$_2$
⑫
FAD
NAD(P)H + H$^+$
⑨
NAD(P)$^+$
Crotonyl-CoA
4-hydroxybutyrate
⑪
ATP + CoA
ADP + P$_i$
4-hydroxybutyryl-CoA
⑩
ATP + CoA
AMP + PP$_i$

Figure 6 The 4-hydroxybutyrate cycle. The enzymes are as follows: 1, pyruvate synthase; 2, phosphoenolpyruvate synthetase; 3, phosphoenolpyruvate carboxylase; 4, malate dehydrogenase; 5, fumarase; 6, fumarate reductase; 7, succinyl-CoA synthetase; 8, succinyl-CoA reductase; 9, succinate semialdehyde reductase; 10, 4-hydroxybuturyl-CoA synthetase; 11, 4-hydroxybutyryl-CoA dehydratase; 12, crotonyl-CoA hydratase; 13, 3-hydroxybutyryl-CoA dehydrogenase; and 14, acetoacetyl-CoA β-ketothiolase.

the acetyl-CoA pathway, the reductive citric acid cycle, the 3-hydroxypropionate cycle, or the 4-hydroxybutyrate cycle for CO_2 assimilation.

In *P. abyssi* and *P. occultum*, there are low levels (5–15 nmol min^{-1} mg^{-1} cell protein) of RubisCO activity, which is the key enzyme of the Calvin cycle. For *P. abyssi*, RubisCO activity increases approximately twofold per 10 °C temperature increase, as expected for most enzymes, and activity requires strictly anoxic and reducing conditions. The product of the reaction is 3-phosphoglycerate. However, ribulose-5-phosphate kinase activity is not measured, suggesting that CO_2 assimilation does not occur via the standard Calvin cycle. Similarly, RubisCO activity is present in several Euryarchaeota including methanogens, the hyperthermophilic heterotrophs in the Thermococcaceae, and *Archaeoglobus fulgidus*. All lack ribulose-5-phosphate kinase activity. Ribulose-1,5-bisphosphate is generated in *M. jannaschii* using 5-phospho-D-ribose-1-pyrophosphate (PRPP) as a substrate, which is an intermediate in nucleotide biosynthesis. A similar pathway is found in *T. kodakaraensis* where adenosine monophosphate is used as the starting material instead of PRPP. First the adenine in AMP is replaced with a phosphate to form ribose-1,5-bisphosphate, then an isomerase converts this to ribulose-1,5-bisphosphate. Although pathways for CO_2 assimilation via RubisCO seem to exist in Euryarchaeota, they do not appear to contribute significantly, if at all, to CO_2 assimilation.

Acetate catabolism

Acetate is potentially an important carbon source in high-temperature environments. It is a common metabolite formed by heterotrophs during the breakdown of organic material and is the primary end product of acetogens. The most common pathway of acetate catabolism in bacteria is via the glyoxylate shunt. First, acetate and CoA are combined to form acetyl-CoA by acetyl-CoA synthase using the energy of ATP and forming AMP + PP_i (**Figure 3**). Then some of the acetyl-CoA condenses with oxaloacetate to form citrate and enters the citric acid cycle. Isocitrate is formed from citrate. At this point, the fate of the carbon varies. Some isocitrate remains within the citric acid cycle for biosynthesis reactions. The remainder is cleaved into succinate and glyoxylate by isocitrate

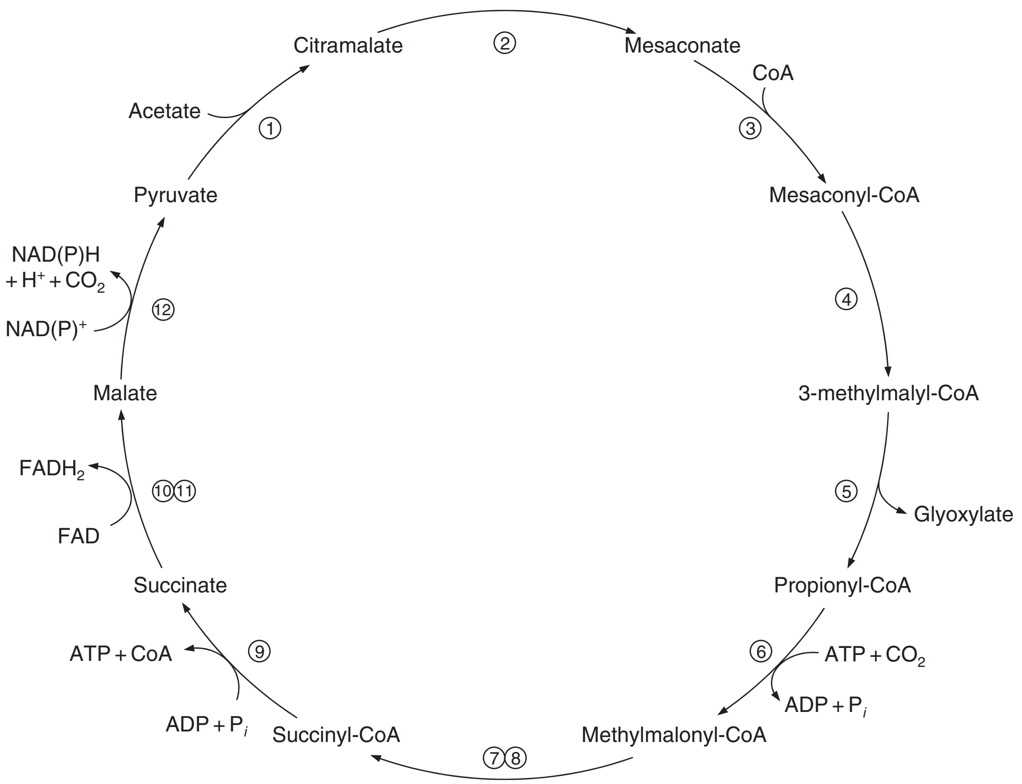


Figure 7 The citramalate cycle. The enzymes are as follows: 1, citramalate synthase; 2, citramalate dehydratase; 3, mesaconyl-CoA synthetase; 4, mesaconyl-CoA hydratase; 5, 3-methylmalyl-CoA lyase; 6, propionyl-CoA carboxylase; 7, methylmalonyl-CoA epimerase; 8, methylmalonyl-CoA mutase; 9, succinyl-CoA synthetase; 10, succinate dehydrogenase; 11, fumarase; and 12, malic enzyme.

lyase. The succinate then re-enters the citric acid cycle pool of intermediates. Glyoxylate is then combined with a second molecule of acetyl-CoA to form malate by malate synthase. Malate can either enter the citric acid cycle pool for biosynthesis reactions or be used to form pyruvate using the malic enzyme. The key enzymes of the glyoxylate shunt are isocitrate lyase and malate synthase, and both of these as well as the complete citric acid cycle are found in the thermoacidophilic archaeon *Sulfolobus acidocaldarius*.

Other thermophilic organisms lack isocitrate lyase and pyruvate synthase activities, the two most common means for biosynthesis from acetyl-CoA, when grown on acetate. In these cases, acetate catabolism is accomplished using the citramalate cycle (**Figure 7**). Acetate (or acetyl-CoA) combines with pyruvate to form citramalate by citramalate synthase. After a series of enzyme reactions, 3-methylmalonyl-CoA is cleaved to form propionyl-CoA and glyoxylate. The glyoxylate can be used to form malate using acetyl-CoA and malate synthase. Propionyl-CoA is carboxylated and eventually forms intermediates of the citric acid cycle using enzymes found in the 3-hydroxypropionate and citric acid cycles. Pyruvate is recycled from an intermediate in the citric acid cycle. The citramalate cycle was first described in purple bacteria, which grow best on acetate when H_2, CO_2, and low levels of either pyruvate or organic compound are added to the growth medium. Similarly, the hyperthermophilic archaeon *P. islandicum* increases its citramalate synthase and 3-methylmalyl-CoA lyase activities when grown on acetate relative to autotrophic and heterotrophic growth, suggesting that is uses the citramalate cycle in part for acetate metabolism. It grew best on acetate when H_2 and low levels of yeast extract (0.001%) were added to the medium.

The biochemical overlap between the citric acid cycle, the glyoxylate shunt, the 3-hydroxypropionate cycle, and the citramalate cycle highlights the importance of viewing these and other pathways holistically because it is likely that they do not always operate completely independent of the others. Furthermore, these pathways each perform multiple cellular functions for the cell. Some or all of the citric acid cycle is involved in all of the pathways listed above and also functions for energy production and biosynthesis reactions such as amino acid synthesis. Portions of the 3-hydroxypropionate cycle are also used for propanoate metabolism and the steps of the citramalate cycle are also used for leucine biosynthesis. These overlaps may have significant evolutionary implications and their study is important to understand the natural history of catabolic and anabolic pathways.

Heterotrophy

The majority of high-temperature microorganisms are heterotrophs or facultative autotrophs (**Table 1**). Not surprisingly, the most common organic compounds catabolized at high temperatures are carbohydrates and peptides. However, some thermophiles and hyperthermophiles also oxidize low-molecular-weight organic acids and many thermophiles catabolize hydrocarbons as sources of carbon and electrons.

Carbohydrate metabolism

Carbohydrate metabolism can be divided into four categories: uptake, hydrolysis, glycolysis, and gluconeogenesis. The enzymes for gluconeogenesis are found in most organisms, including autotrophs, whereas those for uptake, hydrolysis, and glycolysis vary. Many of the hyperthermophilic enzymes involved in glycolysis and gluconeogenesis are biochemically and phylogenetically unique to either hyperthermophiles or archaea (**Figure 8**). For example, glucokinase and phosphofructokinase from *P. furiosus* are ADP-dependent rather than ATP-dependent and do not show any sequence similarity with their ATP-dependent counterparts in mesophilic bacteria. Glyceraldehyde-3-phosphate is oxidized to 3-phosphoglycerate in a single enzyme step with concomitant reduction of ferredoxin rather than NAD^+ and is catalyzed by the unique tungsten-containing protein glyceraldehyde-3-phosphate oxidoreductase. Both $NAD(P)^+$-dependent glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase are present and are homologous to their counterparts in mesophilic bacteria, but they are used for gluconeogenesis rather than for glycolysis in *P. furiosus.* Furthermore, fructose-1,6-bisphosphate aldolase, fructose-1,6-bisphosphatase, and phosphoglucose isomerase are all unique to either hyperthermophiles or archaea.

Three membrane-bound sugar-binding proteins have been characterized from *P. furiosus* that are specific for maltose/trehalose (MalE), maltose/maltodextrin (MBP), and cellobiose (CbtA). These demonstrate the specificity and coordination of carbohydrate uptake in hyperthermophiles. The MalE binding, permease (MalFG), and ATP-binding transporter (MalK) proteins from *Thermococcus litoralis* and the operon encoding these proteins were characterized as well as the regulatory protein (TrmB) for this operon, and showed that the sequences, function, and regulation of ATP-binding cassette (ABC)-type transport systems in hyperthermophiles are similar to those found in mesophiles. The *malEFGK* operon in *P. furiosus* is flanked by insertion sequences but is absent in *P. abyssi* and *Pyrococcus horikoshii*, suggesting lateral gene transfer from other organisms. Using proteomics, MalE and CbtA were identified in the membrane cellular fraction of *P. furiosus* grown on a mixture of maltose and peptides, and MalE is one of the most abundant proteins within the membrane.

The genes for MBP and CbtA in *P. furiosus* are likewise part of an ABC-type operon but lack flanking insertion sequences and are found in *P. abyssi* and *P. horikoshii.* Furthermore, the maltodextrin uptake operons in *P. furiosus* and *Pyrobaculum aerophilum* contain the gene encoding for the sugar hydrolase amylopullulanase (*apu*), whereas the cellobiose operon in *P. furiosus* is next to and shares a putative promoter region with the β-mannosidase gene (*bmn*), thus demonstrating a tight coupling between sugar uptake and hydrolysis in these organisms. *P. furiosus* amylopullulanase is an extracellular glycosylase that is active at temperatures up to 140 °C. This demonstrates that some proteins are stable well above 110 °C and that the biogenic impact of *P. furiosus* in its native environment extends beyond its maximum growth temperature as its extracellular enzymes ‘forage’ for growth substrates.

Peptide metabolism

Peptide metabolism can be divided into three categories that are functionally similar to those found in carbohydrate metabolism: uptake, hydrolysis, and peptidolysis (**Figure 9**). Unlike the sugar ABC transport system, little is known about the peptide ABC transport system in hyperthermophiles. Using proteomics, a putative membrane dipeptide-binding protein was highly abundant in the membrane fraction of *P. furiosus* cells grown on tryptone and maltose along with the MalE-binding protein. Up to 13 protease activity bands are observed in gelatin-containing zymograms from *P. furiosus* cell extracts, demonstrating the large suite of proteases available with the cells. Four of the 13 predicted transaminases in *P. furiosus* were shown to have varying degrees of specificity for amino acids, although each uses 2-oxoglutarate as the amine group acceptor. The glutamate produced from the transamination reaction is recycled back to 2-oxoglutarate by glutamate dehydrogenase with concomitant reduction of $NADP^+$ in *P. furiosus.* Hyperthermophiles and archaea produce up to four ferredoxin-linked 2-keto acid oxidoreductases that decarboxylate the acid, pass electrons to ferredoxin, and ligate CoA to the remaining compound (**Figure 8**). Three of these (IOR, VOR, and OGOR) are unique to archaea. The coenzyme is then cleaved, forming an organic acid with the phosphorylation of ADP to ATP, which is the only substrate-level phosphorylation step within the peptidolysis pathway.

P. furiosus growth on maltose was compared with its growth on peptides using growth kinetics, metabolite analyses, enzyme activities, and DNA microarray analyses. Based on growth rates, *P. furiosus* grows better on peptides than on maltose. As expected, the primary organic acid produced when cultures are grown on maltose is acetate (**Figure 7**), whereas growth on peptides yields a fairly

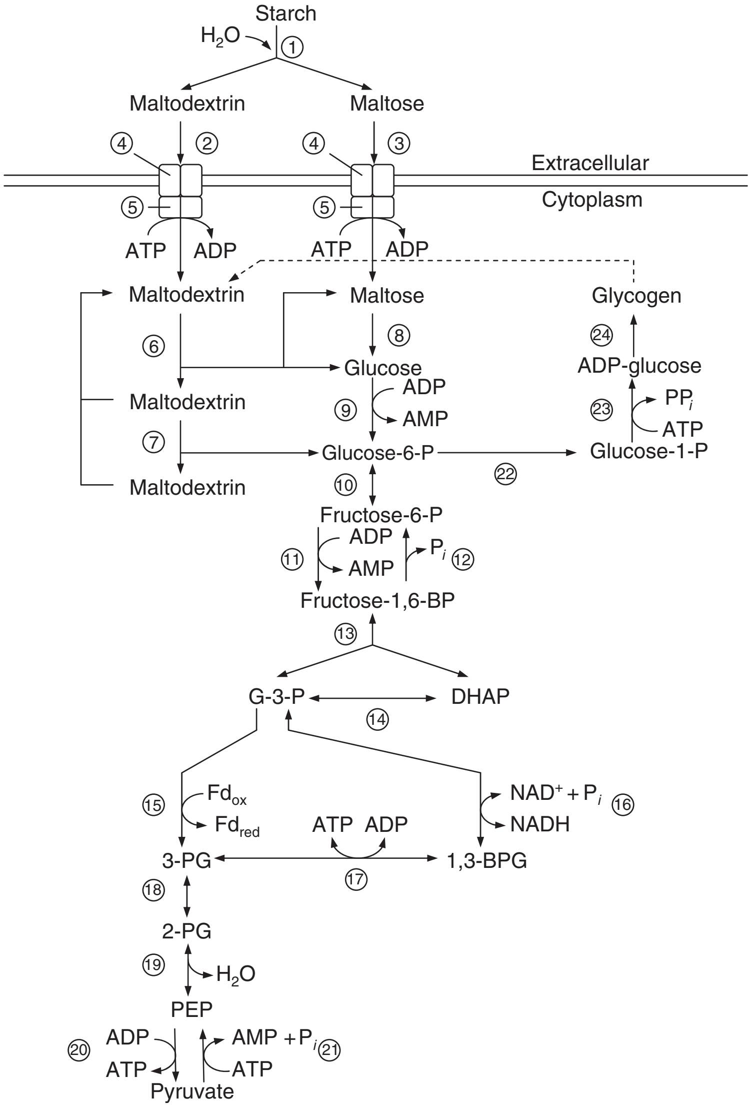

Starch
H_2O
①
Maltodextrin
Maltose
④
②
④
③
Extracellular
Cytoplasm
⑤
ATP
ADP
⑤
ATP
ADP
Maltodextrin
Maltose
Glycogen
⑥
⑧
㉔
Glucose
ADP-glucose
ADP
Maltodextrin
⑨
AMP
㉓
PP_i
ATP
⑦
Glucose-6-P
Glucose-1-P
Maltodextrin
⑩
㉒
Fructose-6-P
ADP
⑪
AMP
P_i
⑫
Fructose-1,6-BP
⑬
G-3-P
DHAP
⑭
Fd_{ox}
⑮
Fd_{red}
ATP ADP
$NAD^+ + P_i$
⑯
NADH
3-PG
1,3-BPG
⑰
⑱
2-PG
⑲
H_2O
PEP
⑳
ADP
ATP
AMP + P_i
㉑
ATP
Pyruvate

Figure 8 Starch hydrolysis, uptake, and glycolysis via the modified Embden–Meyerhof pathway and steps for gluconeogenesis. The enzymes are as follows: 1, amylopullulanase; 2, maltose/maltodextrin-binding protein; 3, maltose/trehalose-binding protein; 4, sugar transport permease; 5, sugar transport ATPase; 6, α-amylase; 7, α-glucan phosphorylase; 8, α-glucosidase; 9, glucokinase; 10, glucose-6-phosphate isomerase; 11, fructose-6-phosphate kinase; 12, fructose-1,6-bisphosphatase; 13, fructose-1,6-bisphosphate aldolase; 14, triosephosphate isomerase; 15, glyceraldehydes-3-phosphate:ferredoxin oxidoreductase; 16, glyceraldehydes-3-phosphate dehydrogenase; 17, 3-phosphoglycerate kinase; 18, 3-phosphoglycerate mutase; 19, enolase; 20, pyruvate kinase; 21, phosphoenolpyruvate synthetase; 22, phosphoglucomutase; 23, ADP-glucose synthase; and 24, glycogen synthase. Fd, the electron carrier ferredoxin.

even mixture of acetate, phenylacetate, (iso)butyrate, and isovalerate (**Figure 9**). The activities of glutamate dehydrogenase, 2-oxoglutarate oxidoreductase, indolepyruvate oxidoreductase, isovalerate oxidoreductase, formaldehyde oxidoreductase, aldehyde oxidoreductase, acetyl-CoA synthetase I, glyceraldehydes-3-phosphate dehydrogenase, and cytoplasmic hydrogenase were all significantly higher when *P. furiosus* cultures were grown on peptides. Conversely, the activities of glyceraldehydes-3-phosphate oxidoreductase, acetolactate synthase, and α-amylase were higher when cultures were grown on maltose. In both cases, these enzymes appear to follow their proposed physiological functions. Formaldehyde oxidoreductase, aldehyde oxidoreductase, and glyceraldehyde:ferredoxin oxidoreductase are tungsten-containing enzymes and explain in part why hyperthermophiles generally have a tungsten requirement for growth.

Respiration

The consumption of electron donors and the reduction of terminal electron acceptors are among the primary

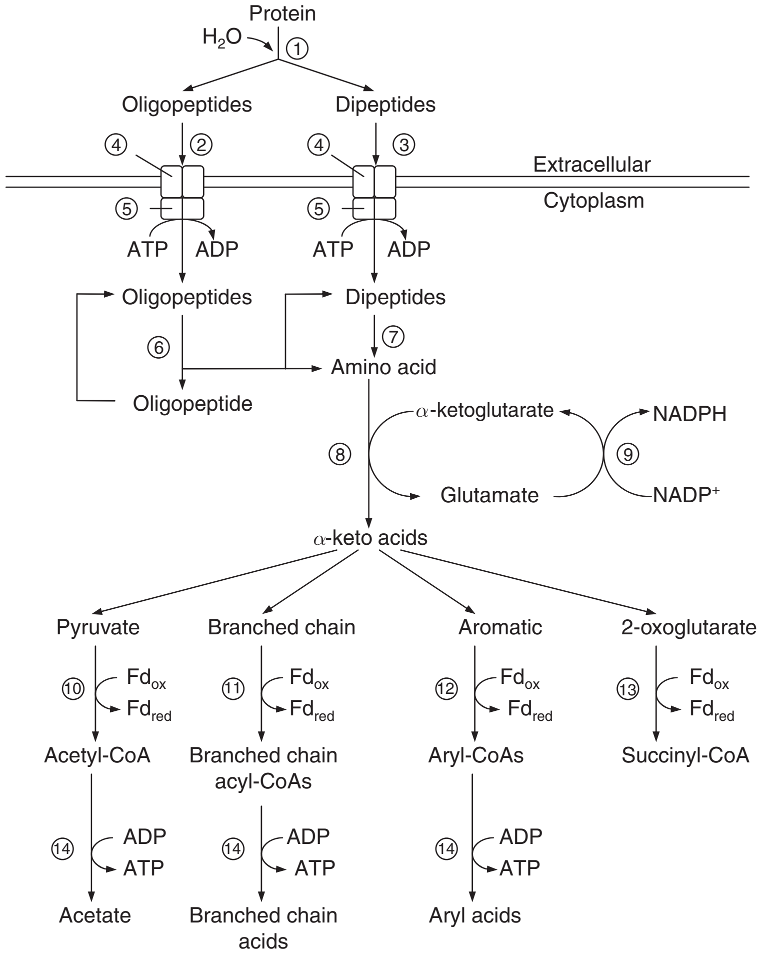

Protein
H_2O
①
Oligopeptides
Dipeptides
④
②
④
③
Extracellular
Cytoplasm
⑤
⑤
ATP
ADP
ATP
ADP
Oligopeptides
Dipeptides
⑥
⑦
Amino acid
Oligopeptide
α-ketoglutarate
NADPH
⑧
⑨
Glutamate
$NADP^+$
α-keto acids
Pyruvate
Branched chain
Aromatic
2-oxoglutarate
⑩
⑪
⑫
⑬
Fd_{ox}
Fd_{red}
Acetyl-CoA
Branched chain acyl-CoAs
Aryl-CoAs
Succinyl-CoA
⑭
ADP
ATP
Acetate
Branched chain acids
Aryl acids

Figure 9 Peptide hydrolysis, uptake, and peptidolysis in archaea. The enzymes are as follows: 1, pyrolysin; 2, oligopeptide-binding protein; 3, dipeptide-binding protein; 4, peptide transport permease; 5, peptide transport ATPase; 6, intracellular protease; 7, prolidase; 8, amino acid aminotransferase; 9, glutamate dehydrogenase; 10, pyruvate:ferredoxin oxidoreductase; 11, α-ketoisovalerate:ferredoxin oxidoreductase; 12, indolepyruvate:ferredoxin oxidoreductase; 13, 2-oxoglutarate:ferredoxin oxidoreductase; and 14, acyl-CoA synthetase. Fd, the electron carrier ferredoxin.

means that microorganisms have of altering the chemistry of their environment. Although several compounds can serve as electron donors for hyperthermophiles, the most common compounds used for this purpose in geothermal environments are H_2, organic compounds, and reduced sulfur compounds. Hydrogen is typically oxidized on the membrane by a hydrogenase where electrons then enter the electron transport pathway. Organic compounds are oxidized as described in the section titled ‘Carbohydrate metabolism’ and ‘Peptide metabolism’ and result in the production of reduced ferredoxin and NADH.

Respiration is a series of exergonic redox reactions within the cytoplasmic membrane that are coupled with proton translocation across the membrane, which forms an electrochemical gradient (**Figure 10**). This proton motive force is then used to generate ATP from ADP and phosphate using a membrane-bound ATP synthase. The canonical electron transport chain through the membrane typically begins with the oxidation of NADH by a membrane-bound NADH:quinone oxidoreductase and the direct reduction of a quinone. Often electrons from the quinone are transferred to a cytochrome *c* by a quinol:cytochrome *c* oxidoreductase, typically a bc_1 complex. Electrons from either the quinones or the cytochrome are then passed to a terminal reductase that reduces the terminal electron acceptor. The marvelous aspect of respiration in bacteria and archaea is that the system is modular, and individual components (e.g., terminal reductases) can be exchanged with changes in environmental conditions and electron acceptor availability.

Homologues of NADH:quinone oxidoreductases are found in the genome sequences of most thermoacidophilic and hyperthermophilic archaea. The catalytic (NuoD) and quinone-binding (NuoH) subunits are conserved except for NuoH in methanogens, but all archaea lack

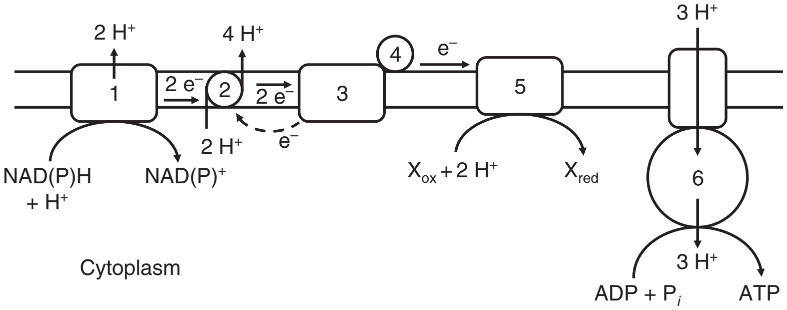


Figure 10 Membrane electron transport pathway. The components are as follows: 1, NADH:quinone oxidoreductase; 2, quinone; 3, quinol:cytochrome *c* oxidoreductase (*bc₁* complex); 4, cytochrome *c*; 5, generic terminal reductase; and 6, H^+-translocating ATP synthase.

the NuoAEFGJK subunits found in bacteria, which includes the NADH binding, flavin, and iron–sulfur cluster containing subunits (NuoEFG). Membrane-soluble archaeal electron carriers have been found in the hyperthermophiles *P. islandicum* and *Pyrobaculum organotrophum*, in the thermoacidophile *S. solfataricus*, and in the mesophilic methanogen *Methanosarcina mazei*. Menaquinones were found in the two *Pyrobaculum* species whereas two novel sulfur-containing quinone-like compounds were observed in *S. solfataricus*. The methanogen uses a 2-hydroxyphenazine derivative called methanophenazine, which could be reduced by a membrane-bound F_{420} dehydrogenase and oxidized by the membrane-bound enzyme heterodisulfide reductase. The use of methanophenazine by methanogens may explain the absence of the quinone-binding subunit in their NADH:quinone oxidoreductase.

The presence of *bc₁* complexes in hyperthermophilic and thermoacidophilic archaea is relatively scarce. They are found in some *Pyrobaculum*, *Aeropyrum*, *Sulfolobus*, and *Acidianus* species, all organisms with some capacity for aerobic growth. Homologues of membrane-bound H^+-translocating ATP synthase are found in the genome sequences of all thermoacidophilic and hyperthermophilic archaea. The catalytic subunits (AtpAB) are conserved, but all archaea lack the AtpGHJK subunits found in bacteria.

Reduction of sulfur compounds

The reduction of elemental sulfur is one of the most common traits of thermoacidophiles and hyperthermophiles (**Table 1**). Elemental sulfur is the terminal electron acceptor for neutrophilic heterotrophs from marine environments (e.g., *Pyrococcus* and *Thermococcus*) and terrestrial environments (e.g., *Thermoproteus* and *Pyrobaculum*), for chemolithoautotrophs from marine environments (e.g., *Pyrodictium*), and for some thermoacidophiles from terrestrial environments (e.g., *Acidianus*). This is not surprising given the abundance of sulfur compounds in their native geothermal environments. Environmental conditions significantly influence the form of sulfur available for respiration. Above pH 5, sulfide anion (HS-) is a nucleophile that reacts with the elemental sulfur ring (S_8) forming polysulfide (S_4^{2-} and S_5^{2-}). Above pH 7 and 75 °C, elemental sulfur disproportionates into thiosulfate and sulfide ($S_8 + 6H_2O \rightarrow 2S_2O_3^{2-} + 4HS^- + 8H^+$).

Pyrodictium and *Acidianus* species couple H_2 oxidation with elemental sulfur reduction. They grow at pH 5-8 and pH 1-4, respectively, suggesting that *Pyrodictium* uses polysulfide whereas *Acidianus* uses S_8. In *Pyrodictium*, H_2 oxidation is coupled directly to sulfur/polysulfide reduction in a membrane-bound multienzyme complex with both hydrogenase and sulfur reductase activities. It contains Fe, Ni, Cu, acid-labile sulfur and hemes *b* and *c* but lacks Mo and W. Quinones were required for activity in the *P. brockii* complex but not in the *P. abyssi* complex, suggesting that the complete electron transport chain is contained within the latter complex. Dissimilatory sulfur reductase from *Acidianus ambivalens* is a heterotrimer with a 110 kDa catalytic subunit containing a molybdo-*bis*-molybdopterin guanine dinucleotide (MGD) cofactor and one Fe-S center, an Fe-S electron transfer subunit, and a membrane anchor. The catalytic subunit contains a twin arginine (Tat) signal peptide sequence, suggesting that it faces the outside of the cell. Mo, but not W, was found in the solubilized membrane. *Sulfolobus* quinone is used to shuttle electrons from a membrane-bound hydrogenase to the sulfur reductase.

The hyperthermophilic archaeon *P. furiosus* can reduce elemental sulfur when it is separated from the cells by a porous barrier and can use polysulfide as the electron acceptor. *Pyrococcus* and *Thermococcus* differ from *Pyrodictium* and *Acidianus* in that they do not appear to use a membrane-bound sulfur/polysulfide reductase for sulfur respiration, nor do they appear to use quinones or cytochromes as electron carriers. Instead, *P. furiosus* uses a soluble NAD(P)H- and CoA-dependent sulfur reductase whose gene expression increases up to sevenfold when cultures are shifted from growth without elemental sulfur to growth with sulfur. The enzyme is a homodimeric flavoprotein. The mechanism for generating a proton motive force is unknown.

Dissimilatory sulfate, thiosulfate, and sulfite reduction is found in several hyperthermophilic archaea (**Table 1**). Sulfate is reduced in three steps ($SO_4^{2-} \rightarrow APS \rightarrow SO_3^{2-} \rightarrow S^{2-}$) by three enzymes localized in the cytoplasm. The ATP sulfurylase from the hyperthermophilic archaeon *Archaeoglobus fulgidus* is a homodimer and activates sulfate using ATP, yielding adenosine phosphosulfate (APS) and pyrophosphate. This and the ATP sulfurylase from the thermophilic bacterium *Thermus* contain a zinc site that is absent in mesophiles, suggesting that it may be related to thermostability at higher temperatures. Dissimilatory APS reductase from *A. fulgidus* is a heterodimer that contains FAD and two Fe-S clusters. Dissimilatory sulfite reductase from *A. fulgidus* has an $\alpha_2\beta_2$ structure and contains siroheme iron, nonheme iron, and acid-labile sulfide. It uses six electrons to reduce sulfite to sulfide. Our understanding of the source of electrons for these reductases and their relationship with the development of a proton motive force is at a rudimentary level.

Dissimilatory thiosulfate reduction occurs on the membrane producing sulfite and sulfide, and then the sulfite is reduced in the cytoplasm to sulfide as described above. The amount of thiosulfate reductase in the membrane fraction of *P. islandicum* cultures increased dramatically in thiosulfate-grown cultures relative to those grown on elemental sulfur and iron. Like the sulfur/polysulfide reductases described above, the thiosulfate reductase in *P. islandicum* is predicted to be a membrane-bound heterotrimer with MGD and Fe-S cofactors. Dissimilatory sulfite reductase from *P. islandicum* has biochemical properties that are nearly identical to those of *A. fulgidus*.

Reduction of nitrogen compounds

Denitrification is found in a limited number of hyperthermophilic archaea (**Table 1**). Nitrate is reduced in four steps ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) by four enzymes. In contrast to denitrifying bacteria, all four denitrifying enzymes in the hyperthermophilic archaeon *P. aerophilum* are membrane bound and use menaquinol as an electron donor. Dissimilatory nitrate reductase from *P. aerophilum* is a heterotrimer that consists of a 146 kDa catalytic subunit with an MGD cofactor and one Fe-S center, an electron transfer subunit with four Fe-S centers, and a membrane anchor with biheme *b* and quinol-oxidizing capability. Like the sulfur reductase in *A. ambivalens*, the catalytic subunit contains a twin arginine (Tat) signal peptide sequence, suggesting that it faces the outside of the cell, which is unlike bacterial nitrate reductases that face the cytoplasm. If so, this would significantly influence the manner in which *P. aerophilum* generates a proton motive force when grown on nitrate. Cultures did not grow without the addition of tungstate; however, concentrations above 0.7 μmol l^{-1} led to a fourfold decrease in dissimilatory nitrate reductase activity. Therefore, tungsten does not replace molybdenum in this metalloenzyme as it does in other thermophiles but apparently is required by other enzymes in the organism. Variations in tungstate concentrations had no effect on nitrite reductase and NO reductase activities. NO reductase from *P. aerophilum* is homomeric, contains derivatives of heme *b*, and uses menaquinone as an electron donor. Denitrification to N_2O was also measured in *Ferroglobus placidus*.

Oxygen

The majority of thermophiles and especially hyperthermophiles are anaerobes, due in large part to the insolubility of O_2 in water at high temperatures and the lack of fluid contact with O_2. However, there are several organisms that are obligate aerobes, microaerophiles, or facultative anaerobes (**Table 1**). As expected, these organisms are generally found in geothermal environments such as in hot springs that interface with oxic environments. Aerobic respiration generally requires electrons carried by cytochrome *c* that are passed to O_2 via cytochrome *c* oxidase. Various forms of this enzyme are found in *Aeropyrum* and *Pyrobaculum* whereas quinol oxidases are found in *Sulfolobus* and *Acidianus*. *Pyrobaculum oguniense* has both cytochrome *a* and cytochrome *o* containing heme-copper oxidases. The *bc₁* complex and the cytochrome *o*-containing oxidase are present in the membranes of cells grown aerobically and anaerobically whereas the cytochrome *a*-containing oxidase is only present in aerobically grown cells. The two oxidases have different affinities for O_2 and are specialized for microaerophilic and aerobic growth.

Metal compounds

Two forms of ferric iron are generally used for growth of bacteria and archaea: soluble Fe(III) that is chelated with citrate and insoluble Fe(III) oxide hydroxide (FeO). Several hyperthermophiles grow on FeO whereas only *Pyrobaculum* and *Geoglobus* grow on Fe(III) citrate. Often the end product of FeO reduction is insoluble magnetic iron. *P. islandicum* also can reduce U(VI), Tc(VII), Cr(VI), Co(III), and Mn(IV). Frequent research questions with mesophilic dissimilatory iron-reducing bacteria are whether they are able to reduce FeO without direct mineral contact and whether polyheme *c*-type cytochromes are required. The two most commonly studied iron-reducing bacteria are *Shewanella* and *Geobacter*. Both require polyheme *c*-type cytochromes for iron reduction. *Shewanella* can grow without direct FeO contact by producing an extracellular electron shuttle whereas *Geobacter* requires direct contact unless a soluble mediator is provided. *P. aerophilum* and *Pyrobaculum arsenaticum* can grow without direct FeO contact whereas

P. islandicum and *Pyrobaculum calidifontis* require direct contact. Genome sequence analyses show that *P. aerophilum*, *P. islandicum*, and *P. arsenaticum* lack polyheme *c*-type cytochromes whereas *P. calidifontis* contains a cytochrome with eight predicted hemes that is highly homologous to those found in *Shewanella* and *Geobacter*. Growth of *P. aerophilum* and *P. islandicum* on Fe(III) citrate and FeO is favored at pHs slightly above neutral and at reduction potentials that are above −220 mV. In contrast, growth of *P. islandicum* on thiosulfate and elemental sulfur is favored at slightly acid pHs and at low reduction potentials (−570 mV). Growth of *P. aerophilum* on nitrate is favored at neutral pH and at reduction potentials above −220 mV.

H_2 production

The anaerobic catabolism of organic compounds often yields low molecular weight organic compounds (e.g., acetate) and H_2. Although common, H_2 production ($E_o' = -410$ mV) by most bacteria is easily inhibited due to their use of NADH ($E_o' = -320$ mV) as the electron donor for the redox reaction (thermodynamically, the midpoint potential (E_o') of the electron donor should ideally be more negative than that of the electron acceptor). Therefore, this process often requires the presence of a H_2 syntroph such as a methanogen in order to keep H_2 at low partial pressure. In contrast, *Pyrococcus* and *Thermococcus* readily produce H_2 as their primary metabolite when grown in the absence of elemental sulfur, their preferred terminal electron acceptor, without a H_2 syntroph. The electron donor for H_2 production in *P. furiosus* is ferredoxin ($E_{m,95\,^\circ C} = -471$ mV), making the reaction more energetically favorable. The hydrogenase from *P. furiosus* is membrane bound and receives electrons directly from ferredoxin. The reaction is coupled directly with proton translocation across the membrane and the development of a $\Delta\Psi$ and a ΔpH. ATP synthesis on the membrane was likewise shown to be linked to H_2 production. *P. furiosus* also has two cytoplasmic hydrogenases that use NADH as the electron donor, which are upregulated when cultures are grown without sulfur.

Relationship between Organisms and their Environment

The high temperatures and geochemistry found in terrestrial and marine geothermal sites are unique. Volcanically derived gases and products from water–rock reactions support chemolithoautotrophic-based microbial communities in what has been termed the deep, hot biosphere. Endolithic microbial communities are pervasive in these environments and likely contribute significantly to subsurface biomass production, which may constitute a significant portion of the total biomass on the planet. The subsurface biosphere is a largely unknown and untapped natural resource. Thermophiles and hyperthermophiles inhabit these environments and serve as model organisms for microbial processes that occur at high *in situ* temperatures. Although known hyperthermophiles may comprise only a small minority of the total microbial population in a geothermal environment, their metabolisms are likely reflections of the kinds of processes occurring within them. Because they are typically not found in nongeothermal background fluids, they can serve as tracers of *in situ* chemical and physical conditions within geothermal environments.

Before one can use these organisms as models of biogeochemical processes in geothermal environments, there are a number of fundamental questions that must be addressed related to the relationship between high-temperature organisms and their environment. For example, what are the physical and chemical constraints on metabolic processes? Are different forms of thermophile and hyperthermophile metabolism spatially and temporally segregated on the basis of fluid chemistry? Clearly, the presence of thermoacidophiles, thermoneutrophiles, and thermoalkaliphiles shows how pH can influence microbial distributions and metabolisms, but can these types of changes be observed on a finer scale even within the same organism? What are the different ways in which organisms assimilate CO_2 or respire a given compound? Are these differences rooted in environmental factors that favor one metabolism over another? Many hyperthermophiles have a requirement for tungsten to meet the needs of certain enzymes found in central metabolic pathways. Are there other unique cofactors used by these organisms? What do these mean with respect to the natural history of these organisms?

In conclusion, extremophiles from hot environments have moved from mere curiosity to a group of organisms that have significant medical and biotechnological applications and are useful for the study of the evolution and biochemistry of metabolic pathways and the biogeochemistry of geothermal environments. Many thermophiles and most hyperthermophiles belong to the Archaea, which is the third superkingdom of life for which there is still much to be learned. Because physiology and ecology go hand in hand, the continued study of high-temperature organisms from these two perspectives should expand our appreciation for these organisms and the function they have in nature.

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Fermentation

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Glossary

anaerobic respiration An anaerobic type of metabolism in which organic (and in a special case also inorganic) compounds are degraded and external electron acceptors other than oxygen are used.

electron transport-coupled phosphorylation ATP synthesis by the membrane-bound ATP synthase with the electrochemical gradient across the cytoplasmic membrane as the driving force.

fermentation An anaerobic type of metabolism in which organic compounds are degraded in the absence or without the use of external electron acceptors. A mixture of oxidized and reduced metabolites is produced and secreted by cells.

fermentation balance The sum of the oxidized and reduced compounds produced as end products during fermentation in which the oxidation state is calculated in arbitrary units.

reducing equivalents Hydrogen or electrons withdrawn from the substrate during oxidative breakdown.

substrate-level phosphorylation A phosphorylated organic compound that is generated as an intermediate during substrate degradation is used for the synthesis of ATP.

Y_{ATP} Cell mass in grams dry weight obtained from 1 mol of ATP upon growth on a monomeric substrate in minimal medium.

Abbreviations

ALS	acetolactate synthetase
CAP	Catabolite activation protein
Fd	flavodoxin
FNR	Fumarate–nitrate–reductase regulatory protein
GAP	glyceraldehyde-3-phosphate
IHF	Integration host factor
KDPG	2-keto-6-phosphogluconate
LDH	lactate dehydrogenase
NADH	nicotinamide adenine dinucleotide
PC	pyruvate carboxylase
PDC	pyruvate decarboxylase
PEP	phosphoenolpyruvate
PGA	6-phosphogluconate
PK	pentose phosphoketolase
POR	pyruvate-ferredoxin oxidoreductase

Defining Statement

Fermentation, which was originally defined by Louis Pasteur as "life without oxygen," is now understood in biochemical terms as the degradation of organic compounds in the absence or without the implication of external electron acceptors. This review describes the mechanisms via which fermenting organisms maintain their redox balance, and it gives an account of different pathways of fermentation and how facultative organisms manage to switch their energy metabolism between fermentation and respiration.

Introduction

Many environments on the surface of earth are devoid of oxygen, either because it is consumed in chemical reactions or by the respiratory activity of aerobic organisms. This creates anoxic macroenvironments such as lake sediments, or microenvironments such as soil particles in which anaerobic organisms can thrive. Instead of using oxygen as the terminal acceptor for the electrons withdrawn from the substrate during degradation, these anaerobes use alternate acceptors such as nitrate, nitrite, sulfur, or sulfate in a process designated anaerobic respiration. If such acceptors are not available, selected organisms can carry out fermentations in which internally produced electron acceptors are used. The range of substrates that can be degraded via fermentation is broad and includes carbohydrates, amino acids, short-chain organic acids, purines, pyrimidines, and a few others. Fermentation can be an obligatory process, as in the diverse group of clostridia, which lack the ability to switch to aerobic or anaerobic respiration when the relevant electron acceptors are provided, or it may be facultative, as in staphylococci, enterobacteria, or yeast, which have complex regulatory mechanisms to ensure that fermentative enzymes are produced only when the cells are deprived of external electron acceptors like oxygen. Some groups of obligatory fermenting organisms degrade their substrates also in the presence of oxygen.

Fermentation as a metabolic process needs to be differentiated from the use of this term in biotechnology, which denotes the production of any biological product in a bioreactor, also under aerobic conditions.

Fermentation Balances

The average energy yield of fermentations lies in the range of 2–2.5 mol of ATP per mole of consumed glucose. Because of this low-energy gain, most of the carbon of the substrate is not incorporated into cell mass but rather appears in the fermentation end products. Because the reducing equivalents withdrawn from the substrate must be transferred to an internally generated acceptor, the sum of the oxidation state of the reduced products and of the oxidized products must equal the oxidation state of the substrate. Fermentations, therefore, are disproportionation reactions when one neglects the fact that only a minor part of the carbon is incorporated into cell mass. The calculation of a fermentation balance, therefore, can give important clues about the metabolic routes via which the products are formed and may shed light on the question of whether an unknown oxidant or reductant participates. An example is shown in **Figure 1(a)**.

An important role in the fermentation of many organisms is played by hydrogenases. These enzymes either reduce protons with electrons to molecular hydrogen or oxidize hydrogen in the reverse reaction. In fermentations, hydrogen production, that is, the use of protons as internal electron acceptors, helps to dissipate the reducing equivalents and thus enables a shift in the ratio of reduced carbon products to the oxidized side. Because molecular hydrogen has a low solubility in water, its formation pulls the kinetics of unfavorable reaction

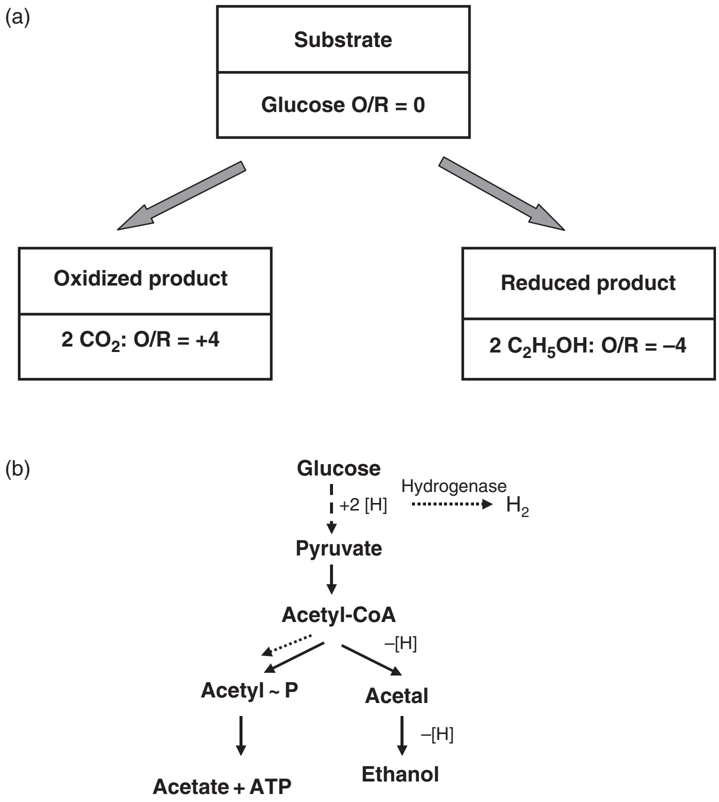

Figure 1 (a) Fermentation balance of the ethanol fermentation. In the calculation, oxygen is arbitrarily assigned a value of +1, hydrogen of −0.5, and nitrogen of +1.5. (b) Effect of the reduction of protons by hydrogenase on the conversion of acetyl-CoA to acetate and ethanol.

equilibria, especially when hydrogen-producing organisms live in syntrophy with hydrogen-consuming ones. **Figure 1(b)** displays the role of hydrogenases during the conversion of acetyl-CoA either into acetate plus ATP or into ethanol, reactions occurring during many types of fermentations. In contrast to acetate generation, ethanol formation requires 2 mol of reducing equivalents, which are supplied during the degradation of 1 mol of glucose in glycolysis; consequently, acetate and ethanol are formed in equimolar amounts. The withdrawal of reducing equivalents into the hydrogenase reaction causes a shift of the balance to the side of acetate, which is advantageous because of the increased gain of ATP.

Fermentation of Carbohydrates

Carbohydrates in the form of starch, cellulose, or hemicellulose are the most abundant naturally occurring electron donors, and thus are rich sources of energy for the growth of heterotrophic organisms. Their hydrolytic breakdown into the monomeric or oligomeric constituents provides important fermentation substrates for many obligate and facultative anaerobes. Processes in nature where metabolic breakdown of organic material by fermentation takes place include the rumen and the guts of animals and humans or the degradation of plant material in compost or anaerobic soil compartments. Fermentation as a metabolic process, however, also plays a significant role in biotechnology, as up to around 90% of the substrates may be converted into secreted products. The production of bulk chemicals such as ethanol, lactate, or butanol relies on the fermentative activity of microorganisms as well as on the production of major sources of human nutrition such as fermented milk products and vegetables, or of animal feed in the form of silage. Fermentation reactions also contribute to the spoilage of food, and they play a role in the infection process of organisms such as staphylococci or *Bacteroides*, which enable these organisms to settle in anoxic niches of the human body. This practical relevance is dealt with in ‘Diary products’, ‘Fermented foods’, ‘Food Spoilage, Preservation and Quality Control’, ‘Lactic Acid, Microbially Produced’, and ‘Solvent Production’.

Propanediol Fermentation: The Substrate Glycerol is Converted in a 1:1 Ratio into an Oxidant and a Reductant

The question whether an organism is able to degrade a carbohydrate monomer by fermentation depends on its ability either to induce pathways or to use constitutively expressed pathways that accept the reducing equivalents set free during the oxidative breakdown of the substrate. An interesting example in this respect presents the aerobic and anaerobic breakdown of the polyol glycerol by *Escherichia coli* and by *Klebsiella* species. *E. coli* and *Klebsiella* are able to degrade glycerol by aerobic and anaerobic respiration, as they possess aerobic and anaerobic dehydrogenases that convert glycerol-3-phosphate into dihydroxyacetone phosphate (**Figure 2(a)**, top). Both enzymes are membrane-bound flavoproteins that feed the electrons directly into the respective aerobic or anaerobic respiratory chains. A third enzyme capable of this interconversion is an oxidoreductase whose function lies in the formation of glycerol-3-phosphate for lipid biosynthesis. None of these three enzymes is able to support fermentation as they are functionally connected either to respiratory chains or they serve biosynthetic purposes. Glycerol fermentation, however, can only proceed in *Klebsiella* because it is able to induce the formation of two functional and unique branches of glycerol breakdown (**Figure 2(b)**). The oxidative branch initiates with the oxidation of glycerol to dihydroxyacetone by glycerol dehydrogenase; subsequent phosphorylation of dihydroxyacetone delivers an intermediate of the central glycolytic route and allows ATP generation via substrate-level phosphorylation. This oxidative branch produces an extra reducing equivalent as compared with the breakdown of the C3 unit of glyceraldehyde-3-phosphate (GAP). To balance the redox state of glycerol fermentation, an additional mole of glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol (diol) dehydratase to 3-hydroxypropionaldehyde whose aldehyde group is a ready acceptor of electrons resulting in nicotinamide adenine dinucleotide (NADH) reoxidation under 1,3-propanediol formation. Thus, 50% of the glycerol consumed is released as propanediol, an interesting bulk chemical for polymerization reactions. Another product of this pathway of biotechnological interest is dihydroxyacetone, a chemical used in creams for the bronzing of skin.

Pyruvate or Derivatives Thereof Serve as Electron Acceptors

Types of carbohydrate fermentation are classified in most cases according to the products that are formed and released into the medium. **Figure 3** schematically presents the pathways followed by some prominent fermentation systems. A characteristic feature of most carbohydrate breakdown routes during fermentation is that pyruvate plays a central role. Altogether six enzyme systems can be differentiated that are involved in the conversion of pyruvate, with the aim to either directly reduce it or convert it into intermediates that can accept the reducing equivalents liberated during carbohydrate degradation either through glycolysis or through the Entner–Doudoroff pathway and thus to reoxidize the reduced coenzymes.

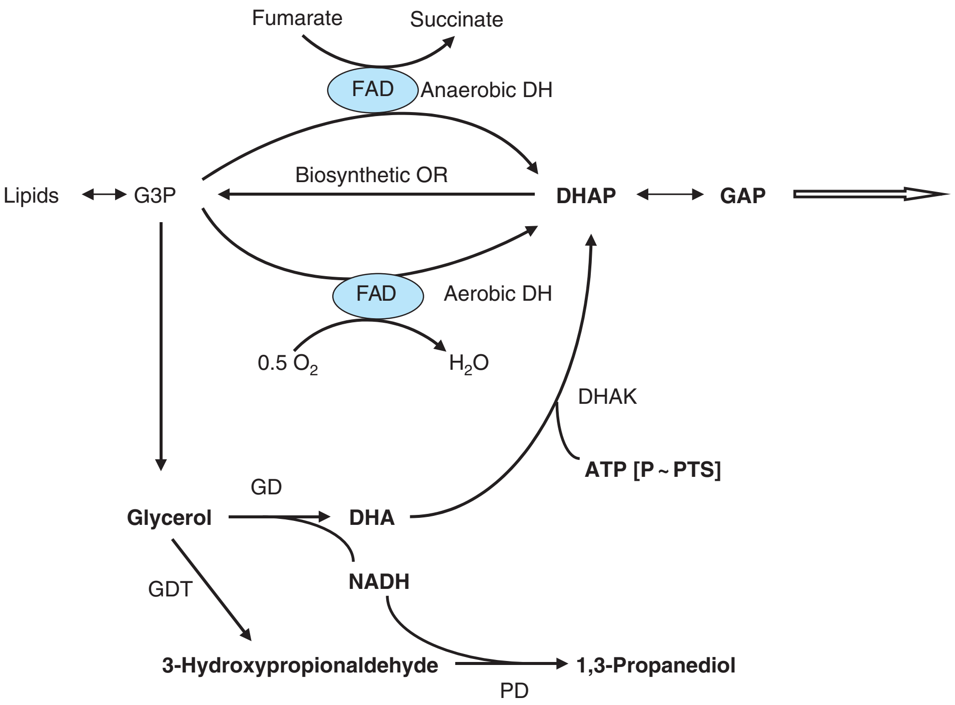

Figure 2 The metabolism of glycerol by *Escherichia coli* and by *Klebsiella*. Glycerol-3 phosphate (G3P) derived from the hydrolysis of fat and of lipids can be oxidized by *E. coli* and *Klebsiella* in the presence of external electron acceptors. An aerobically induced G3P dehydrogenase (DH) and an anaerobically induced isoenzyme feed electrons directly into specific respiratory chains. A third enzyme, an oxidoreductase (OR), is responsible for *de novo* G3P biosynthesis when G3P is not provided in the medium. The lower part of the figure (written in bold letters) presents the oxidative and reductive branches of glycerol fermentation by *Klebsiella*. The homodimeric dihydroxyacetone kinase (DHAK) from *Klebsiella* uses ATP as substrate, whereas the heterotrimeric enzyme from *E. coli* uses the phosphate from PEP, which is transferred via a cascade of relay proteins to the ADP firmly bound to the ultimate kinase subunit. *E. coli* possesses the genetic capacity to synthesize the constituent polypeptides of this pathway except that coding for a functional glycerol dehydratase. Transformation with the gene for glycerol dehydratase enables *E. coli* to also ferment glycerol. DHAK, dihydroxyacetone kinase; GD, glycerol dehydrogenase; GDT, glycerol dehydratase; PD, propanediol dehydrogenase, P ~PTS indicates that the phosphate for dihydroxyacetone phosphorylation in *E. coli* is transferred from PEP via the phosphotransferase system to dihydroxyacetone kinase.

The reduction of pyruvate to lactate is catalyzed by lactate dehydrogenase (LDH) and – depending on the organism – delivers one of the two stereoisomers of lactate, which may be the sole fermentation end product as in homofermentive lactobacteria or produced as one of the several end products as in enterobacteria (see **Figure 4**(**b**)). In alcoholic fermentation by yeast or *Zymomonas*, the substrate for the dissipation of reducing equivalents is acetaldehyde, the product of the decarboxylation of pyruvate by pyruvate decarboxylase (PDC). In propionic acid fermentation by *Propionibacterium* and in succinate formation by enterobacteria, the reductive branch of the citric acid cycle serves as a means to reoxidize the reduced coenzymes. The initial reaction that provides the substrate to be reduced, oxalacetate, is catalyzed by pyruvate carboxylase (PC) in *Propionibacterium* or phosphoenolpyruvate (PEP) carboxylase in enterobacteria. In butyric acid fermentation by clostridia, pyruvate is first oxidized to acetyl-CoA, CO_2, and reduced ferredoxin by pyruvate-ferredoxin oxidoreductase (POR) and 2 mol of acetyl-CoA are subsequently condensed to acetoacetyl-CoA, which delivers butyrate or butanol by investing 2 or 4 mol of reducing equivalents, respectively. As already mentioned (**Figure 1**(**b**)), acetyl-CoA can be converted into acetate via the phosphotransacetylase/acetokinase sequence or reduced to ethanol with the expenditure of two reducing equivalents. A final reductive branch initiating from pyruvate leads to butanediol in the mixed acid fermentation followed by the *Enterobacter*/*Klebsiella* group of enterobacteria. Here, 2 mol of pyruvate are condensed to acetolactate by acetolactate synthetase (ALS) (**Figure** (**4b**)), which is reduced to butanediol via acetoin.

The Carbohydrate Substrate Itself Serves as Electron Acceptor

The fermentation type characteristic of the β-group members of *Lactobacillus* deserves special mention; they degrade glucose according the following overall balance:

$$3\ \text{glucose} \rightarrow 1\ \text{lactate} + 1\ \text{acetate} + 1\ CO_2 + 2\ \text{mannitol}$$

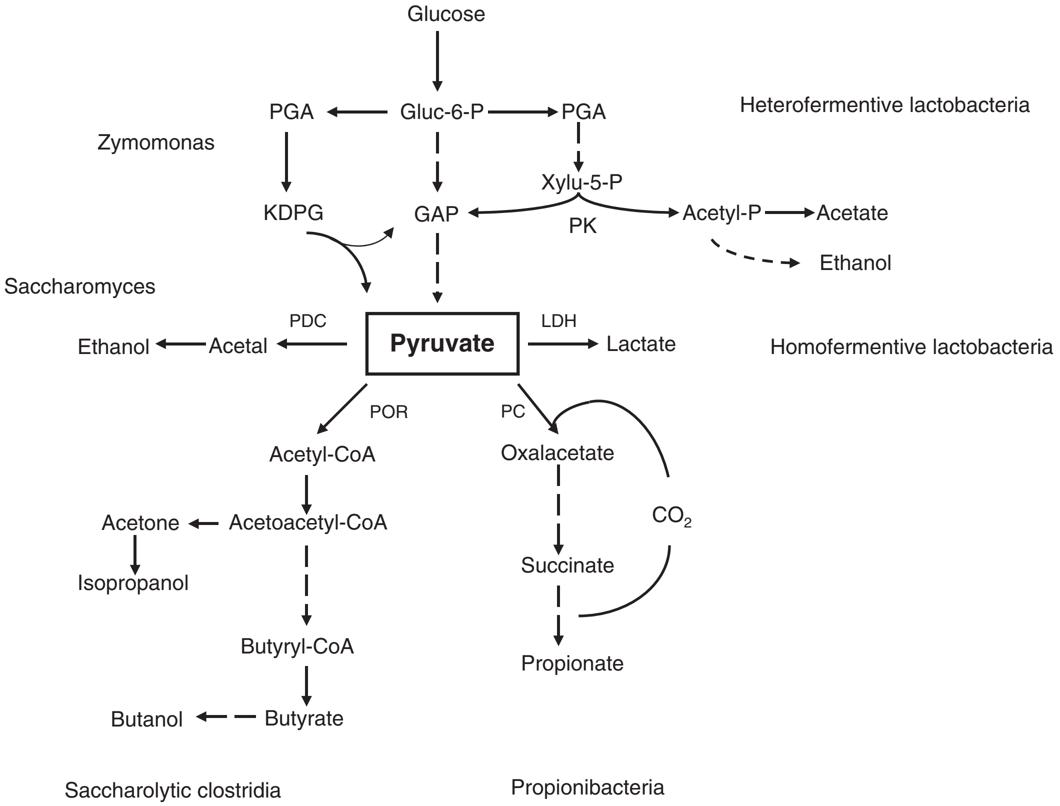
Glucose
Gluc-6-P
PGA
PGA
Zymomonas
KDPG
GAP
Xylu-5-P
PK
Acetyl-P
Acetate
Ethanol
Heterofermentive lactobacteria
Saccharomyces
PDC
Ethanol
Acetal
Pyruvate
LDH
Lactate
Homofermentive lactobacteria
POR
PC
Acetyl-CoA
Oxalacetate
CO_2
Acetone
Acetoacetyl-CoA
Isopropanol
Succinate
Butyryl-CoA
Propionate
Butanol
Butyrate
Saccharolytic clostridia
Propionibacteria

Figure 3 Scheme of carbon flux in selected fermentation types with carbohydrate as the substrate. Only characteristic intermediates are given; the reducing equivalents that are generated or invested are not demonstrated. Gluc-6-P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; KDPG, 2-keto-6-phosphogluconate; LDH, lactate dehydrogenase; PC, pyruvate carboxylase; PDC, pyruvate decarboxylase; PGA, 6-phosphogluconate; PK, pentose phosphoketolase; POR, pyruvate-ferredoxin oxidoreductase; Xylu-5-P, xylulose-5-phosphate. The mixed acid fermentation of enterobacteria is not included (see **Figure 4**).

Here 1 mol of glucose is converted to xylulose-5-P via the hexose monophosphate route, which is then cleaved to acetylphosphate and GAP by pentose phosphoketolase (PK). GAP delivers lactate via the lower branch of glycolysis and the activity of LDH, whereas acetylphosphate is converted to acetate with the gain of 1 mol of ATP via phosphotransacetylase and acetokinase. As the formation of xylulose-5-phosphate is connected with the generation of 2 mol reducing equivalent and as the organism is unable to reduce acetate to ethanol, it needs to search for alternate compounds as acceptor for the surplus electrons: as a consequence, two units of hexose are reduced to mannitol to balance the redox state.

CO_2 as Electron Acceptor

Finally, the fermentation conducted by homoacetogenic bacteria such as *Moorella thermoacetica* that degrade 1 mol of glucose into three acetate units is chosen as an example, as the CO_2 liberated during glycolysis serves as the electron acceptor. Two of the three acetate moieties are generated from the two pyruvates formed during glycolysis. This part of the pathway also delivers two CO_2 molecules and elemental hydrogen, which is the product of proton reduction by the electrons derived from the cleavage of pyruvate. The third one arises from the reduction of the two CO_2 molecules liberated during pyruvate cleavage: one CO_2 molecule is reduced to the methyl group by the activities of a selenium- and tungsten-containing formate dehydrogenase and the attachment to and subsequent reduction at the coenzyme tetrahydrofolate. After transfer to a corrinoid protein, it is carbonylated with CO at CO-dehydrogenase/acetyl-CoA synthase to acetyl-CoA. The reducing power is provided by elemental hydrogen via the activity of a hydrogenase. In summary, the long-time enigmatic formation of the third acetate unit can be visualized as the intracellular reduction of CO_2 with hydrogen as the reductant, whereby both are gaseous products arising from glycolysis.

Fermentation of Organic Acids, Amino Acids, and Purines

Fermentation of Organic Acids

Apart from carbohydrates, short-chain organic acids and amino acids are major substrates for fermentations. These

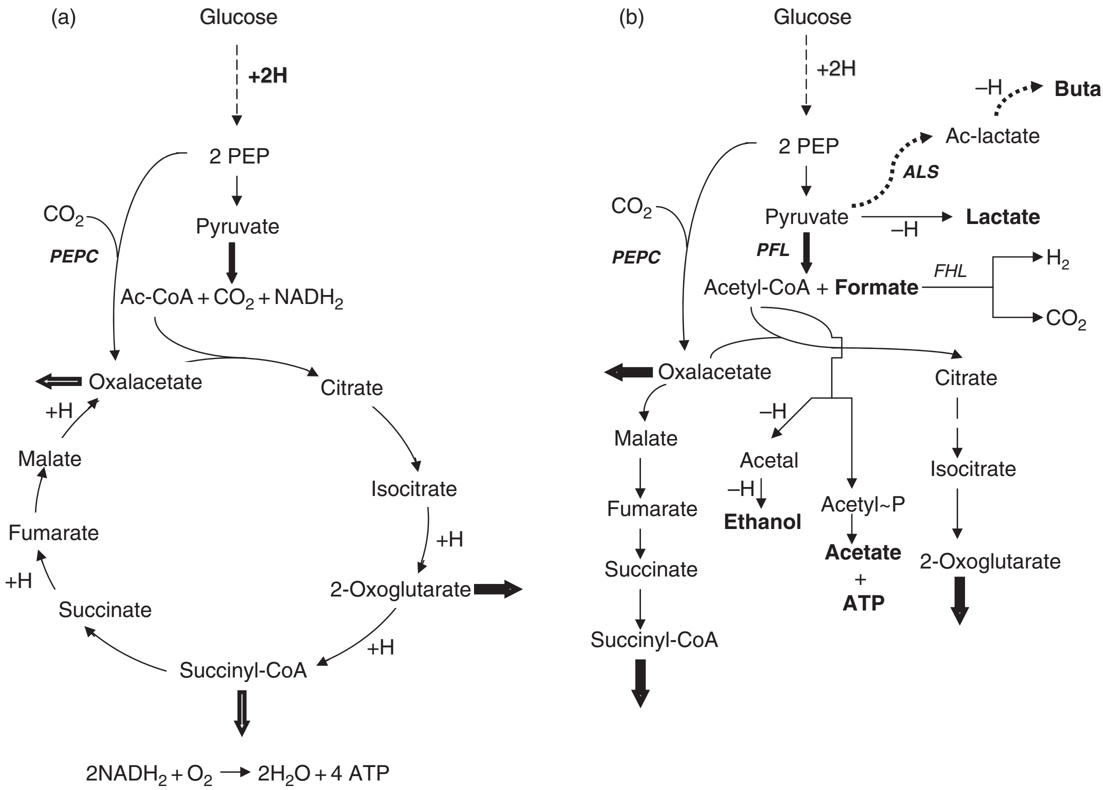
(a)
Glucose
+2H
2 PEP
Pyruvate
CO2
PEPC
Ac-CoA + CO2 + NADH2
Oxalacetate
Citrate
+H
Malate
Isocitrate
Fumarate
+H
+H
Succinate
2-Oxoglutarate
+H
Succinyl-CoA
2NADH2 + O2 → 2H2O + 4 ATP
(b)
Glucose
+2H
–H
Butanediol
2 PEP
Ac-lactate
ALS
CO2
Pyruvate
–H
Lactate
PEPC
PFL
FHL
H2
Acetyl-CoA + Formate
CO2
Oxalacetate
Citrate
–H
Malate
Acetal
Isocitrate
–H
Fumarate
Ethanol
Acetyl~P
Succinate
Acetate
+
ATP
2-Oxoglutarate
Succinyl-CoA

Figure 4 Scheme of carbon flux in *Escherichia coli* and related enterobacteria under fully respiratory (a) and fermentative (b) conditions. (a) Pyruvate is cleaved by pyruvate dehydrogenase (PD) and the citric acid cycle is functional as a cycle. Reducing equivalents generated are denoted by +H. Intermediates withdrawn into anabolism are indicated by bold arrows pointing away from the respective intermediate. The citric acid cycle is replenished by the activity of PEP carboxylase (PEPC) to replace the intermediates withdrawn into biosynthetic routes. (b) Pyruvate is cleaved by pyruvate formatelyase (PFL), and the citric acid cycle is interrupted and divided into two branches because the synthesis of 2-oxoglutarate dehydrogenase is repressed. +H denotes the generation of reducing equivalents, whereas –H assigns its consumption. The pathway functional in the *Klebsiella/Enterobacter* group leading to butanediol is indicated by dotted arrows. ALS, 2-acetolactate synthetase; FHL denotes the activity of the formate hydrogenlyase complex.

organic acids are generated from carbohydrates by the activities of the organisms in the reactions described in the section titled ‘Fermentation of carbohydrates’ or by oxidative deamination of amino acids arising from proteolysis. Among the short-chain organic acids, acetate is by far the most abundant substrate. Its fermentation by methanogenic bacteria such as *Methanothrix* or *Methanosarcina* is widespread in nature and about 70% of the methane produced biologically derives from the reduction of the methyl group of acetate in a quasi-internal disproportionation reaction.

$$CH_3\text{-}COOH \rightarrow CH_4 + CO_2$$

Despite its seemingly simple chemistry, the reaction involves about half a dozen unique coenzymes and cofactors.

The major amount of methane biologically produced from acetate occurs in lake sediments, in tundra soils, rice paddies, and in the rumen of ruminants. Because of its about 20-fold higher potential as a ‘green-house’ gas compared with CO_2, methane formation presently is under particular attention. On the other hand, the development, construction, and optimization of biogas plants in which agricultural materials and municipal wastes are converted into methane-containing biogas gain increasing economic relevance.

Two pathways have convergently evolved for the fermentation of lactate, both of them yield propionate as end product. *Propionibacterium* species oxidize lactate (an end product of lactobacterial fermentations in dairy products) to pyruvate and follow the route along the reductive branch of the citric acid cycle described above. On the other hand, *Clostridium propionicum* forms propionate by the dehydration of lactyl-CoA to acrylyl-CoA and its reduction to propionyl-CoA.

Citrate fermentation has been studied extensively because citrate is the major source for the generation of the typical flavor of fermented milk, in particular of diacetyl. The fermentation of citrate is also a significant diagnostic characteristic to differentiate *Salmonella* from the *E. coli* group of enterobacteria. Citrate degradation is initiated by its cleavage into oxalacetate and acetate by citrate lyase. Oxalacetate decarboxylase is a membrane-bound enzyme complex that catalyzes biotin-dependent pyruvate formation coupled to the extrusion of a sodium ion across the cytoplasmic membrane, causing a buildup of a sodium gradient that can be used for ATP synthesis with the aid of a sodium dependent ATP synthase. The pyruvate formed is nonoxidatively cleaved by pyruvate formatelyase into acetyl-CoA and formate, and further metabolism follows the route of a characteristic mixed acid fermentation (see **Figure 4**). Similar sodium-dependent decarboxylases are involved in succinate or methylmalonate fermentation by *Propionigenium modestum* and in glutamate fermentation by *Acidaminococcus* and *Peptococcus*.

Fermentation of Amino Acids

Amino acids are readily fermented by many bacteria, especially by members of the Gram-positive anaerobes belonging to the peptolytic clostridia such as *Clostridium stricklandii*, *Clostridium litorale*, or *Clostridium histolyticum*, by nonsporogenic *Peptococcus* sp., as well as by *Eubacterium* (*E.*) species such as *E. acidaminophilum*. Because of the nature and occurrence of their substrate, amino acid fermenters are frequently associated with processes of food spoilage and with anaerobic infections. **Table 1** lists examples in which a single amino acid can serve as a substrate of fermentation. Because of the diversity of chemical structures, the fermentation of amino acids, unlike that of most carbohydrates, does not follow a route converging in some central metabolite (like pyruvate in carbohydrate fermentations); instead, it proceeds through individual pathways that involve a plethora of chemically unusual and often also novel reactions. Frequently, different and phylogenetically distant organisms possess different pathways. Examples of this are listed in **Table 1**. Glutamate fermentation by *Clostridium tetanomorphum*, for example, proceeds through mesaconate as an intermediate, whereas that of *Acidaminococcus* and *Peptostreptococcus* involves oxidative deamination to 2-oxoglutarate, reduction and dehydratation to glutaconate, and decarboxylation to crotonyl-CoA. Its dismutation to butyrate and acetate yields the same end products as glutamate fermentation by. *C. tetanomorphum*. Another example of convergently evolved pathways concerns threonine fermentation: By *E. coli*, L-threonine is dehydrated to 2-oxobutyrate, which is the substrate for an isoenzyme of the classic pyruvate formatelyase but with propionate and formate as cleavage products. *C. propionicum*, on the other hand, cleaves 2-oxobutyrate oxidatively by means of a ferredoxin-dependent oxidoreductase.

Many of the peptolytic clostridia and also Gram-negative organisms such as *Treponema denticola* are able to ferment pairs of amino acids in the so-called Stickland reaction. One of the amino acids serves as electron donor whose breakdown usually delivers CO_2, H_2, and organic acids, whereas the other functions as electron acceptor. Preferred electron donors are alanine, the branched chain amino acids, and histidine. Preferred acceptors are glycine, proline, arginine, and tryptophan. With alanine and glycine as Stickland pair, the overall reaction formally proceeds as follows:

$$\begin{gathered}\text{alanine} + 2\ \text{glycine} + 3\text{ADP} + 3\text{P}_i \\ \rightarrow 3\ \text{acetate} + \text{CO}_2 + 3\text{NH}_4 + 3\text{ATP}\end{gathered}$$

The oxidation of the donor involves oxidative deamination to the 2-oxo acid followed by oxidative decarboxylation to the acyl-CoA derivative, which can be used in substrate-level phosphorylation for ATP synthesis via the phosphotransacetylase/acetokinase reaction sequence. The reduction of the acceptor amino acid involves at least three proteins, two of which are selenoproteins. Formally, the amino acid is reduced to the corresponding organic acid plus NH_4^+ with the generation of another molecule of ATP, again by substrate-level phosphorylation:

$$\begin{gathered}\text{glycine} + \text{P}_i^{2-} + \text{ADP}^{2-} + 2\text{e}^- + 3\text{H}^+ \\ \rightarrow \text{acetate} + \text{NH}_4^+ + 3\text{ATP}^{3-} + \text{H}_2\text{O}(-14.4\ \text{kcal mol}^{-1})\end{gathered}$$

Fermentation of Purines and Pyrimidines

Purines and pyrimidnes arise from hydrolytic degradation of nucleic acids; anaerobically, they can be fermented

Table 1 Examples of selected amino acid fermentations

Amino acid	*Overall reaction products*	*Organisms*
Alanine	Acetate, propionate, NH_4^+	*Clostridium propionicum*
Glycine	H_2, NH_4^+, CO_2, acetate	*Peptococcus anaerobius*
Glutamate	H_2, NH_4^+, CO_2, acetate, butyrate	*Clostridium tetanomorphum*
Glutamate	NH_4^+, CO_2, acetate, butyrate	*Acidaminococcus fermentans*
Threonine	H_2, NH_4^+, CO_2, propionate	*C. propionicum*
Threonine	NH_4^+, formate[a], propionate	*Escherichia coli*
Lysine	2 NH_4^+, acetate, butyrate,	*Clostridium subterminale*
Arginine	NH_4^+, CO_2, ornithine	Extreme halophiles, *Pseudomonas*, *Mycoplasma*

[a] Secreted at neutral or alkaline pH.

by a few specialists. Some of them are so restricted in their substrate spectrum that they accept a single substrate only. Thus, *Clostridium acidi-urici* and *Clostridium cylindrosporum* solely ferment guanines, the end products being formate, CO_2, $NH_4{}^+$, acetate, and, in the case of the latter organism, glycine. Organisms that are able to degrade pyrimidines or derivatives thereof are scarce. *Clostridium oroticum*, for example, degrades orotic acid to acetate, CO_2, and $NH_4{}^+$.

C. acidi-urici, *C. cylindrosporum*, and also *Clostridium purinolyticum* couple purine degradation to the reduction of glycine in a Stickland-type reaction. They possess a glycine reductase and glycine is used for the disposal of reducing equivalents that are generated during anoxic degradation of purines. The overall balance can be formulated as follows:

$$\begin{aligned}&\text{formiminoglycine} + 2\text{ADP} + 2\text{P}_\text{i}\\&\quad\rightarrow \text{acetate} + \text{CO}_2 + \text{NH}_4^+ + 2\text{ATP}\end{aligned}$$

Such mixed substrate fermentation may be of considerable importance in the degradation of complex mixtures of natural compounds.

Energy Conservation Reactions in Fermentations

Depending on the availability of external electron acceptors, three different modes of coupling substrate degradation with energy conservation can be differentiated, namely aerobic respiration, anaerobic respiration, and fermentation. Aerobic and anaerobic respirations conserve energy by the buildup of a primary proton motif force across the cytoplasmic membrane, which is used for the synthesis of ATP by electron transport-coupled phosphorylation. In contrast, the classic energy conservation mode in fermentations is substrate-level phosphorylation. Immediate phosphoryl donors with a high group transfer potential are acetyl-phosphate, propionyl-phosphate, butyryl-phosphate, carbamoyl-phosphate, 1,3-diphosphoglycerate, and phosphoenolpyruvate. Fermentative growth on arginine by many Gram-positive and Gram-negative bacteria and by some archaea, for example, relies on the synthesis of ATP using the high-energy anhydride bond of carbamoyl phosphate:

$$\text{arginine} + \text{H}_2\text{O} \rightarrow \text{citrulline} + \text{NH}_4^+$$

$$\text{citrulline} + \text{P}_\text{i} \rightarrow \text{ornithine} + \text{carbamoyl-phosphate}$$

$$\text{carbamoyl-phosphate} + \text{ADP} \rightarrow \text{CO}_2 + \text{NH}_4^+ + \text{ATP}$$

A single mole of ATP, therefore, is formed from 1 mol of arginine. As a consequence, growth on arginine is possible only under the condition that uptake of the substrate does not afford energy. This is accomplished by the function of an energy-neutral antiporter that couples the uptake of arginine to the extrusion of the product ornithine.

Other energy-rich intermediates originating in fermentative pathways are acetyl-CoA, propionyl-CoA, butyryl-CoA, and succinyl-CoA. ATP is generated by CoA exchange between the latter three substrates with acetate and the use of the resulting acetyl-CoA for ATP synthesis via the phosphotransacetylase/acetokinase reaction sequence.

Fermentation is the 'last resort' for energy conservation. It is now generally accepted that Y_{ATP} (the yield of cell mass obtained from 1 mol of ATP after growth on monomeric substrates in minimal salts medium) is about 10.5 g. The determination of Y_{ATP} allowed the conclusion that in most fermentations, no more than 3 mol of ATP per mole of degraded glucose are formed. The ATP that is generated by substrate-level phosphorylation is then used to build up a proton motif force across the cytoplasmic membrane via the cleavage of ATP coupled with the extrusion of protons or sodium ions.

Substrate-level phosphorylation is frequently accompanied by direct membrane energy reactions. Thus, in the case of the sodium-dependent decarboxylation of oxalacetate during citrate fermentation by some enterobacteria or methylmalonate decarboxylation by *Propionigenium*, sodium ions are translocated to the outside of the cell, resulting in the buildup of a sodium gradient used for ATP synthesis. Propionibacteria and enterobacteria conserve energy also by direct electron transport-coupled phosphorylation during the reduction of fumarate in mixed acid fermentations. Finally, in several fermentation routes, the export of certain acidic fermentative end products produced in symport with protons can contribute to the gain of energy.

Regulation of Fermentations

Depending on their mode of energy conservation and on their relation to oxygen, fermenting organisms can be divided into several classes: Obligatory fermenters are unable to switch their metabolism to alternate modes of energy conservation such as aerobic or anaerobic respiration; whereby some classes are inhibited or even killed in the presence of oxygen and others are tolerant to oxygen, at least to concentrations below the atmospheric level. The members of this aerotolerant group comprise most of the genera and species used in food technology or in biotechnological processes. Facultative fermenters, on the other hand, are able to switch to a respiratory type of metabolism using electron transport-coupled phosphorylation for ATP syntheses when they are provided with an external electron acceptor. During the shift to a medium containing such an external acceptor, enzymes with a sole role in fermentative metabolism are repressed in their synthesis.

The regulation of fermentation in such facultative fermenters has attracted considerable attention because it sheds light on the adaptation of microorganisms to environmental conditions like oxygen or pH. They also deserve interest from an applied point of view, because the use of these organisms in biotechnological processes may necessitate the manipulation of the underlying regulatory mechanisms. Most of the regulatory work has dealt with the metabolic consequences of a shift from aerobiosis to anaerobiosis, mainly of *E. coli* and its enterobacterial relatives.

Central Metabolic Routes of *E. coli* During Aerobic Respiration and Fermentation

Figure 4 compares the central metabolic pathways of *E. coli* upon growth under respiratory (a) and fermentative (b) conditions. The major characteristics of aerobic respiration are that pyruvate is cleaved oxidatively by pyruvate dehydrogenase and that the cells contain a functional citric acid cycle. The reduced coenzymes feed their electrons into an aerobic respiratory chain, which in the case of *E. coli* only harbors two coupling sites for ATP generation. Formally, the energy gain is thus 24 mol of ATP per 1 mol of glucose degraded.

There are a number of regulatory events that convert the central pathway characteristic of fully aerobic growth into the mixed acid-type fermentation depicted in **Figure 4**(**b**). First, pyruvate dehydrogenase is no longer synthesized but its activity is replaced by pyruvate formatelyase, which cleaves pyruvate nonoxidatively into acetyl-CoA and formate. Second, the citric acid cycle is interrupted by the repression of the formation of 2-oxoglutarate dehydrogenase. It is thus separated into an oxidative and a reductive branch, which purely serve biosynthetic purposes. Finally, a number of downstream pathways are induced and start to operate to balance the redox status. A LDH is formed that directly reduces pyruvate, an ethanol dehydrogenase is induced, which as a single polypeptide catalyses the reduction of acetyl-CoA first to acetaldehyde and then to ethanol, and at acidic pH values, formate is disproportionated to H_2 and CO_2 by formate hydrogenlyase. Enterobacteria belonging to the *Enterobacter/Klebsiella* group induce the synthesis of a pathway initiated by ALS and leading to butanediol. It competes with pyruvate formatelyase for the substrate pyruvate and thus reduces the formation of acidic products (formate, lactate, acetate, and succnate) characteristic of the fermentation type of *E. coli*. The pathway from acetyl-CoA to acetate plus ATP is constitutive and must function in parallel with an alternate route such as ethanol formation to maintain redox balance (see **Figure 1**). The average energy gain is thus around 2.5 mol of ATP per mole of glucose degraded.

Regulatory Mechanisms Involved in the Metabolic Switch

The fundamental change in the central routes of carbohydrate breakdown in aerobically and anaerobically growing cells described is effected by a set of regulatory circuits. **Table 2** gives a summary of the major ones involved and also lists their physiological targets. The ArcA/ArcB two-component system is responsible for the repression of aerobic enzymes/systems under anaerobic conditions, such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, or the respiratory electron transport chain. Fumarate–nitrate–reductase regulatory protein (FNR) is a transcription activator harboring an Fe/S cluster that senses oxygen; it is required for the activation of genes involved in anaerobic respiration. Catabolite activation protein (CAP) as a transcription activator is a global regulator that binds cyclic AMP and allows the expression of genes for catabolic enzymes that are under repression control of glucose. Integration host factor (IHF) controls the topology of DNA and thus acts as a structural element that modulates gene expression. **Table 2** also lists a number of specific regulators responsible for induction of the expression of the downstream branches of the mixed acid fermentation. They are discussed below.

The key reaction involved in the shift of enterobacteria from respiration to fermentation is the cleavage of pyruvate by pyruvate formatelyase. The enzyme is present in aerobically grown cells, but at a low level only and in an inactive state. Upon shift to fermentative conditions, the enzyme is activated by the introduction of a protein radical located at a glycine residue by means of an activase, with *S*-adenosyl-methionine as cofactor and reduced flavodoxin (Fd) as electron donor (**Figure 5**), according to the following equation:

$$\begin{aligned}&\text{E-H}+S\text{-adenosyl-methionine} + \text{Fd}_{\text{red}}\\&\quad\rightarrow \text{E}^{\circ} + 5'\text{-deoxyadenosine} + \text{methionine} + \text{Fd}_{\text{ox}}\end{aligned}$$

Table 2 Regulatory elements involved in the aerobic–anaerobic metabolic switch in enterobacteria

Regulatory element	*Physiological target*
ArcA/ArcB	Repression of genes involved in aerobic respiration
FNR	Activation of genes involved in anaerobic metabolism
CAP	Catabolite activation protein
IHF	DNA topology
FhlA	Induction of formate regulon genes
Act	Activation of PFL
AdhE	Deactivation of PFL, autoregulation
BudR	Activation of butanediol operon genes

See text for abbreviations.

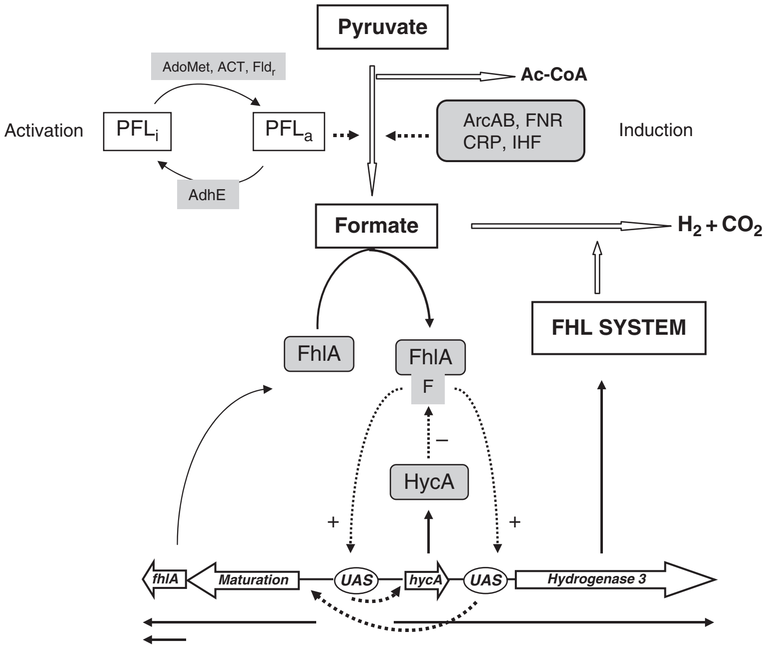

Figure 5 Regulation of carbon flux in the formate regulon of *Escherichia coli*. Regulatory proteins are shaded and their interactions are given by broken arrows. Only relevant genes are indicated. Hydrogenase 3: Genes coding for the structural polypeptides of the hydrogenase components of the formate hydrogenlyase system (FHL). Maturation: genes involved in the maturation of hydrogenase 3. AdoMET, *S*-adenosyl methionine; ACT, Activase; UAS, upstream activatory sequence. For other abbreviations see text.

The introduction of the radical converts the oxygen-stable precursor form into the highly oxygen-sensitive active enzyme. Upon exposure to oxygen, the constituent polypeptide is cleaved at the respective glycine residue located in the C-terminal part of the protein backbone. Concomitant with the activation of the oxygen-stable precursor, about a 15-fold induction takes place.

The *pfl* structural gene lies in a transcriptional unit with *focA*, probably coding for a formate exporter. The gene for the activase is clustered with this transcriptional unit but expressed from its own promoter, warranting the availability of the activating system at any time. The *focA–pfl* operon is transcribed from seven promoters. The trans elements involved in control of the expression are FNR, the ArcA/ArcB two-component regulatory system, the CAP, and the IHF (see **Table 2** and **figure 5**). After full induction, pyruvate formatelyase is the major protein in fermentatively grown cells.

When fermenting cells of *E. coli* encounter oxygen, the oxygen-sensitive radical form of pyruvate formatelyase is rapidly converted into the nonradical oxygen stable but inactive form. Deactivation is accomplished by the AdhE protein, which also catalyzes the reduction of acetyl-CoA to ethanol with acetaldehyde as the intermediate by removing the protein radical. AdhE forms string-like multimers, which have been discovered since long in the electron micrographs of anaerobic cells from *E. coli.*

Regulation of the Downstream Pathways of Mixed Acid Fermentation

As **Figure 4** indicates, acetyl-CoA formed in the anaerobic pyruvate cleaving reaction by enterobacteria can follow two alternative pathways. One of them, to acetate via the phosphotransacetylase-acetokinase route, is coupled to ATP synthesis but does not contribute to NADH reoxidation. The other one, reduction to ethanol, can compensate for this deficiency because two reducing equivalents are consumed. The route to acetate appears to be formed constitutively. On the other hand, the route to ethanol, catalyzed by AdhE, is regulated at the level of gene expression. The AdhE level is expanded considerably under fermentative conditions. Several mechanisms, like the involvement of FruR or the dependence on the NAD/NADH ratio, have been proposed, but there is no definite resolution of the open question yet.

More information, however, is available for the regulation of the metabolism of formate, the second product produced from pyruvate in the pyruvate formatelyase reaction. Under neutral pH conditions, formate is extruded into the medium, potentially serving as a high-energy substrate for one of the two formate dehydrogenases that couple formate oxidation with the reduction of oxygen or of nitrate, if the physiological situation, that is, availability of the terminal electron acceptor, becomes appropriate. Under acidic conditions, the synthesis of the formate hydrogenlyase complex is induced, which disproportionates formate into H_2 and CO_2. Formate hydrogenlyase consists of a formate dehydrogenase (FDHH) and a hydrogenase (hydrogenase 3) component plus several membrane-integral or associated components. The synthesis and the maturation of the complex also include the formation and attachment of the following cofactors: MoCo, the molybdenum cofactor of FDHH, selenocysteine for the formation of the large subunit of FDHH, and the [NiFe] cluster for the active site of the hydrogenase three components. A large set of accessory genes coding for functions in these functions must therefore be coexpressed with the structural genes. Some of them, like the *hyp* genes that have a function in [NiFe] synthesis and ligandation, are coregulated with the structural genes for the formate hydrogenlyase complex and are members of the so-called formate regulon, as formate is the major stimulus.

The expression of these structural genes and maturation genes is under the control of the FhlA protein, which requires formate as a ligand (**Figure 5**). FhlA binds to an upstream regulatory sequence and induces transcription activation at −12/−24 promoters. FhlA thus works like a regulator of two-component systems but, unlike classical regulators of such systems, does not require phosphorylation for activity but rather the binding of the ligand formate.

The structural gene for FhlA is under the control of its own product. Under anaerobic conditions, therefore, a buildup on the cellular level of activator takes place in an autocatalytic manner. Two counteracting mechanisms exist, however, that balance the expression level. The first one consists in the parallel induction of expression of the formate hydrogenlyase genes, which results in lowering the cellular concentration of formate by dissipating it into CO_2 and H_2. The second one relies on the fact that the product of the first gene of the hydrogenase 3 operon (HycA) seems to act as an antagonist of FhlA and to inactivate it, possibly by direct protein–protein interaction.

In summary, the anaerobic expression of *adhE* and of the genes of the formate regulon is subject to different control mechanisms. AdhE formation appears to be controlled by the redox demands of metabolism, whereas the formate hydrogenlyase genes are controlled by the pool level of formate whereby the pH of the medium plays a decisive role. Under neutral pH conditions, formate is extruded into the medium and its energy thus conserved for later oxidation reactions once a terminal electron acceptor like oxygen or nitrate becomes available. Drop of the pH into the acidic range, on the other hand, induces formation of the formate hydrogenlyase system, resulting in the dissipation of formate into the neutral end gaseous products CO_2 and H_2. A major role of the formate hydrogenlyase system may therefore lie in the maintenance of pH homeostasis. Elegantly, the energy is conserved again but in the form of elemental hydrogen.

Such reuse of the fermentation end products when the physiological conditions change seems to be a general principle for organisms that can switch between fermentative and respiratory metabolism. Thus, *E. coli* synthesizes three lactate dehydrogenases, only one of which has a role in fermentation. It is induced under anaerobiosis at acidic pH and converts pyruvate into the D-stereoisomer of lactate. The other two enzymes are membrane-bound flavoproteins that oxidize either D- or L-lactate and feed the electrons into the respiratory chain once an external acceptor has been supplied.

Degradation of organic substrates by fermentation delivers the minimal amount of energy, whereas aerobic respiration offers the maximal energy yield. In addition to oxygen, *E. coli* and its enterobacterial relatives are able to use terminal electron acceptors alternate to oxygen, such as nitrate or nitrite, fumarate, or trimethylamine. Specific primary dehydrogenases like formate dehydrogenase N lead the electrons withdrawn from the substrate via a specific electron transport chain to one of these acceptors, for example, nitrate. Because the redox potential of the nitrate/nitrite pair is less positive than that of the oxygen/hydrogen pair, the gain of energy is lower but still much higher than that available via fermentation. Consequently, regulatory mechanisms have evolved to warrant that fermentation does not take place when nitrate is offered to anaerobic cells. Two main mechanisms are involved. First, the formation of pyruvate dehydrogenase is not switched off fully but only reduced, so it can compete with pyruvate formatelyase for pyruvate and less formate is generated. Second, formate dehydrogenase N is induced in its formation by nitrate and it drains the cellular pool of formate down to a level below the concentration required for activation of FhlA.

Regulation of the Butanediol Formation in *Klebsiella*

Klebsiella and *Enterobacter* species (and also species of the Gram-positive *Bacillus*) can convert 2 mol of pyruvate into 1 mol of butanediol via the intermediates 2-acetolactate and acetoin. This lowers the amount of pyruvate to be cleaved by pyruvate formatelyase and prevents acidification down to those pH levels reached in the mixed acid fermentation of the *E. coli* type. Proceeding in this pathway, however, creates an imbalance in the redox status, as only 1 mol of reducing equivalent from the two generated in glycolysis is reinvested. The imbalance is compensated by the parallel formation of ethanol by the two-step reduction of acetyl-CoA.

The genes for the formation of butanediol are organized in the *bud* operon and their expression is under the control of the BudR regulator, which is an LysR-type regulatory protein. BudR requires acetate as a ligand for activity. Expression is increased at acidic pH values so that one of the physiological roles of butanediol formation may again lie in pH homeostasis.

Regulation of 1,3-Propanediol Fermentation in *Klebsiella*

The anaerobic breakdown of glycerol by *Klebsiella* has attracted attention both from a basic and from an applied interest. Dihydroxyacetone is in use as a means for skin bronzing in cosmetics and 1,3-propanediol is a promising bifunctional molecule for the synthesis of polymers and polyesters. Moreover, glycerol dehydratase is one of the paradigm enzymes for the study of B_{12}-dependent enzyme reaction mechanisms. The genes (*dha*) for the enzymes of the oxidative and reductive branch are induced upon growth on glycerol and subject to catabolite repression by glucose. The actual inducer is dihydroxyacetone, which binds to the transcription activator protein DhaR. Glycerol dehydrogenase is further regulated by posttranslational inactivation when fermenting cells are shifted to aerobiosis.

A unique and novel mode of interaction between catalysis and regulation has been recently experienced for the dihydroxyacetone kinase from *E. coli.* In contrast to the heterodimeric enzyme of *Klebsiella*, which uses free ATP as the phosphor donor, the kinase from *E. coli* consists of three different soluble subunits that transfer the phosphate from PEP and the phosphotransferase system via a phospho-relay protein to an ADP firmly bound to one of the kinase subunits. Two of the kinase subunits also are involved in interaction with the transcription activator DhaR, whereby one acts as coactivator and the other as corepressor. The inability of many *E. coli* strains to degrade glycerol fermentatively is due to a genetically inactivated glycerol dehydratase. Transformation with the respective gene from *Klebsiella* repairs the deficiency.

Regulation of Butyrate–Butanol Fermentation in *Clostridium acetobutylicum*

C. acetobutylicum is an obligatory fermenting organism that degrades carbohydrates into acetate, butyrate, CO_2, H_2, and, depending on the physiological conditions, also acetone and butanol. Because of the potential industrial interest, a considerable amount of work has been invested in increasing the yield of solvents in the butyrate–butanol–acetone fermentation. When the pH is kept at neutral or slightly alkaline values, the organism predominantly produces a mixture of acetate and butyrate, in addition to CO_2 and H_2. When the pH is allowed to drop to values between 4 and 5, acetone and butanol are formed and the amount of butyrate is concomitantly reduced. Solventogenesis appears to be another mechanism maintaining pH homoeostasis and thereby preventing lethal acidification. Similar to the mixed acid fermentation by enterobacteria, it is unknown how the pH is sensed and how the signal is transduced to the genes that are to be controlled.

Manipulation of Fermentation Pathways in Biotechnology

A number of different modules of this encyclopedia deal with the exploitation of fermentations for the synthesis of chemicals or for the processing of human food, animal feed, or for anaerobic treatment of waste. Therefore, in the context of this article whose aim is to concentrate on the general characteristics of fermentations, only a few principal approaches will be discussed that are feasible to improve the substrate spectrum, the rate of degradation, and the yield and purity of the end product.

A major problem for the application of fermentations in biotechnology is the frequently very limited substrate spectrum of the organisms. This may relate to the lack of hydrolytic enzymes, which necessitates the pretreatment of the substrate either chemically or enzymatically or to the inability to degrade disaccharides like lactose or of the pentoses arising from the hydrolysis of hemicellulose. Recently, considerable success has been achieved by the construction of recombinant strains that harbor the relevant genes for hydrolases from heterologous sources. Frequently, the genes coding for the uptake or metabolism of such monomers/oligomers produced by the recombinant hydrolases in the medium must also be introduced.

Economic optimization of fermentation processes often demand the input of cheap substrates in the form of hydrolysates of natural materials such as hemicellulose or lignocellulose. The degradation of all compounds of the mixture, which normally follows a hierarchical order, is desirable; it can again be achieved by the introduction of heterologous genes coding for enzymes with altered substrate affinity and activity or control of synthesis. In other cases, as in the fermentative synthesis of propanediol, the expensive substrate glycerol has been replaced by glucose by combination of the pathway from glucose to glycerol in yeast with the bacterial route from glycerol to propanediol.

Increase of the flux from the substrate to the end products is much more difficult to achieve because it needs, among others, the information on the kinetic constants of the individual reactions, on their equilibria, on substrate and product inhibitions or limiting substrate and coenzyme concentrations. Although this is a timely field of metabolic engineering and systems biology, the approach followed until now is mostly empirical. An elegant project in this respect presents the conversion of the mixed acid fermentation of *E. coli* into a pure ethanologenic fermentation. Key enzymes such as pyruvate formatelyase in the mixed acid fermentation were replaced by anaerobically expressed pyruvate dehydrogenase to improve the pressure on NADH formation by the AdhE enzyme if the yield of ethanol is to be increased. Pathways of the reactions leading to the other products of the mixed acid fermentation that compete with ethanol formation had to be genetically blocked off. Yield increase, especially in solventogenic organisms, also required the isolation of mutants with a higher tolerance against the product or the continuous removal of the product.

Finally, actively fermenting strains have a high reducing power; this can be exploited in enzymatic synthesis procedures by offering a chiral synthone to an organism, which is then hydrogenated and converted into some pure optical isomer, depending on the stereospecifity of the enzyme.

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Flagella, Prokaryotic

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Defining Statement
Structure
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Further Reading

Glossary

flagellar basal body The major structure of the flagellar motor, consisting of ring structures and a rod.

flagellar motor A molecular machine that converts the energy of proton flow into the rotational force.

master genes Flagellar genes regulating the expression of all the other flagellar genes, sitting at the top of the hierarchy of flagellar regulons.

polymorphic transition Interconversion of helical forms on a flagellar filament in a discrete or stepwise manner.

type III export system One of the protein secretion systems that does not use the general secretory pathway. It consists of many protein components.

Abbreviations

GSP	general secretory pathway
HAP	hook-associated protein
PMF	proton motive force
SPI	*Salmonella* pathogenesis Island
T3SS	type III secretion system

Defining Statement

The flagellum is an organelle of bacterial motility. It consists of several substructures – the filament, the hook, the basal body, the C ring, and the C rod. The flagellar motor, an actively functional part of the flagellum, can generate torque from proton motive force. In this article, I will describe mainly the structural aspects of the flagellum revealed in pursuit for the identity of the flagellar motor.

Structure

Filament

The flagellum is a complex structure composed of many different kinds of proteins. However, the term flagellum often indicates the flagellar filament only since the filament is the major portion of the entire flagellum. Especially, in earlier papers, the term flagella always denoted filaments. In this section, I am going to describe the filament and may occasionally call the filament flagellum.

Number of flagella per cell

The number and location of flagella on a cell is one of the readily discernible traits for the classification of bacterial species. The number ranges from one to several hundreds depending on the species, and hence the nomenclature: monotrichous (one) or multitrichous (two or more). Occasionally, the term ‘amphitrichous’ is used for two flagella.

There are three possible locations on a cell body for flagella to grow: polar (at the axial ends of the cell body), lateral (at the middle of the cell body), or peritrichous (anywhere around the cell body). In some cases, ‘lateral’ is used as the counterpart of ‘polar’, as in the two flagellar systems of *Vibrio alginolyticus*: polar sheathed flagellum and lateral plain flagella. The ‘lateral’ flagella have been sometimes mistaken as a part of ‘peritrichous’ flagella, but now there are a several lines of evidence that these two are different from each other: (1) they belong to different flagellar families (see ‘Three flagellar families’); (2) the gene organization of the two is different (see ‘Flagellar genes’); and (3) lateral flagella are inducible in higher viscosity environments, but peritrichous flagella are not. Thus, lateral flagella seem to have its own manner for localization on the cell. A tuft of flagella growing from a pole is called lophotrichous. In most cases, flagella can be named by a combination of number and location; for example, polar lophotrichous flagella of *Spirillum volutans*.

Although ordinary flagella are exposed to the medium, some flagella are wrapped with a sheath derived from the outer membrane (e.g., *Vibrio cholerae*, *Helicobacter pylori*). In an extreme case such as spirochaetes, flagella are confined in a narrow space between the outer membrane and the cell membrane and thus are called the periplasmic flagella or axial filaments. The flagella still can rotate; the helical cell body works as a screw, and the flagella counterbalance the torque on the cell body.

Filament shape and polymorphic transition (Figure 1)

Filament shape is helical. In theory, there are two types of helices, right-handed and left-handed; in reality, *Salmonella* spp. have left-handed filament and *Caulobacter crescentus* has a right-handed filament. However, it should be noted that shapes of these two helices are not mirror images of each other.

There are several detailed filament shapes, and it will be convenient to use the names of typical shapes found in *Salmonella* spp.: normal (left-handed), curly (right-handed), coiled (left-handed), semicoiled (right-handed), and straight. The helical parameters of these helices are discrete and distinguishable from one another.

Flagella can switch between a set of helical shapes under appropriate conditions; not only helical pitch but also helical handedness is interchangeable. The transformation of shapes can be induced by physical perturbation (torque, temperature, pH, salt concentration of medium, etc.). Genetical changes such as point mutations in the flagellin (the component protein of the flagellar filament) gene also result in transformation of helices, but some mutant flagella such as straight flagella have no freedom to transform into another helix.

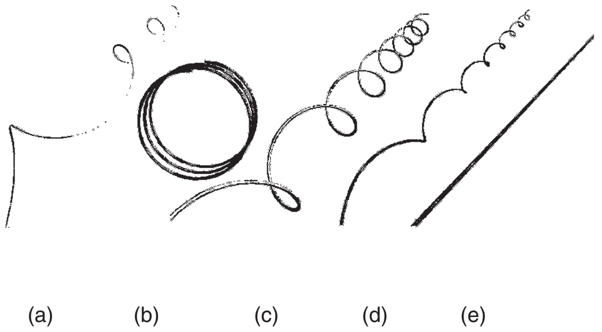

Figure 1 Helical forms of flagellar filaments. Helices are seen from a position slightly off its axial direction so that the handedness can be easily visualized. The figure shows five typical forms with their helical parameters (p: pitch, d: diameter) – (a) normal ($p = 2.55\,\mu$m, $d = 0.6\,\mu$m), (b) coiled ($p = 0$, $d = 1.0\,\mu$m), (c) semicoiled ($p = 1.26\,\mu$m, $d = 0.5\,\mu$m), (d) curly ($p = 1.20\,\mu$m, $d = 0.2\,\mu$m), and (e) straight filament ($p = \infty$, $d = 0$).

This phenomenon called 'polymorphism' of flagella is a visible example of conformational changes in proteins and, therefore, has evoked an idea of a functional role of flagella in motility; could polymorphism of the flagellum by itself cause the motion? The answer is No. Flagella are passive in terms of force generation. Polymorphism of flagella is observed to occur naturally on actively motile cells with peritrichous flagella. The helical transformation is necessary for untangling a jammed bundle of tangled flagella. When normal flagella in a jammed bundle are transformed into curly flagella, knots of tangled flagella run toward the free end of each flagellum to untangle the jammed bundle.

Models that explain the polymorphism were first introduced by Sho Asakura in 1970 and theoretically strengthened by Chris R. Calladine in 1978. Twisting and bending a cylindrical rod give rise to a helix. Models predict 12 shapes, and 8 of them have been found in existing filaments: straight with a left-handed twist, f1, normal, coiled, semicoiled, curly I, curly II, and straight with a right-handed twist. Only a small energy barrier seems to lie between two neighboring shapes. Polymorphic transition occurs from one shape to its neighbors; for example, in a transition from normal to curly I, the filament briefly takes on coiled and semicoiled forms.

Three flagellar families (Figure 2)

The bacterial flagella transform its typical shape into several distinguishable helical shapes (polymorphs) under various environmental conditions as mentioned above. Therefore, we have regarded flagella from other species as one of those polymorphs defined for *Salmonella typhimurium*. Recently, we have found that curly filament of the polar flagellum was not right-handed as expected but left-handed, which urged us to reexamine all flagella shapes so far studied. Indeed, it turned out that the two types of flagella form two distinguishable families.

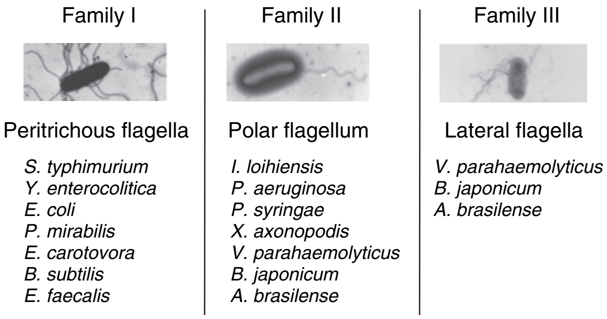

Figure 2 Flagellar family. According to the helical parameters, flagella are divided into three families: Family I for peritrichous flagella, Family II for polar flagella, and Family III for lateral flagella.

A helix is uniquely defined by three parameters: the pitch (*p*), the helix diameter (*d*), and the handedness. If the handedness was expressed as + (right handed) or − (left handed) of the pitch value, any helices will be plotted on the pitch–diameter (p–πd) plane. For example, the parameters of the normal filament are written as (−2.55, 1.88) and those of the curly filament as (+1.20, 0.63). Note that πd is better than *d*; if a tube was flattened, a unique *d* disappears but πd (periphery) remains constant. Polymorphs of *Salmonella* flagellum stay on a circle in the pitch–diameter (p–πd) plot, indicating that they all belong to one family predicted by the Calladine model. In 2005, the flagellar polymorphs of *Idiomarina loihiensis* (Family II) were turned out much smaller than the conventional flagellar family of *S. typhimurium* (Family I). The pitch and diameter of Family II flagella are half of the same of Family I flagella. Furthermore, lateral flagella had helical parameters much smaller than those of the two families and thus belonged to a new family (Family III).

Flagellin

The component protein of the filament is called flagellin. Although the flagellum of many bacteria is composed of one kind of flagellin, some flagella consist of more than two kinds of closely related subspecies of flagellin. The molecular size of flagellin ranges from 20 to 60 kDa. Enterobacteria tend to have larger molecules, while species living in fresh water have smaller molecules. The three-dimensional structure of the flagellin in the filament of *S. typhimurium* has been solved at the atomic level.

One of the most characteristic features of flagellin is evident even in the primary structure of the molecule; the amino acid sequences of both terminal regions are well conserved, whereas that of the central region is highly variable even among species or subspecies of the same genera. As a matter of fact, this hypervariability of the central region gives rise to hundreds of serotypes of *Salmonella* spp.

The terminal regions are essential for binding of each molecule to another to polymerize into a filament. Complete folding of flagellin occurs during assembly; although the terminal regions do not take on any specific secondary structure in solution, they are converted into α-helix upon polymerization.

In the filament, the terminal regions are located at the innermost radius of a cylindrical structure, while the central region is exposed to the outside. It should be noticed that the filament are extremely stable; it does not depolymerize in water, in contrast to actin filaments or tubulin filaments which depolymerize in the absence of salts.

The description of the flagellin molecule is not applicable to that found in archaea. Archaic flagella seem to have a system totally different from bacterial flagella; archaic flagellins have signal sequences, suggesting that the flagellum might grow from the proximal end in the outer membrane, in contrast to the distal growth of bacterial flagellum (see ‘Morphological pathway’).

Flagellin can be posttranslationally modified. *Salmonella* flagellin is methylated at several Lys residues by methylase encoded by *fliB* gene neighboring *fliC* gene. The role of methylation of flagellin is not clear, because *fliB* mutants behave like the wild-type. *Pseudomonas syringae* flagellin is glycosylated at six Ser residues. Deglycosylation of the flagellin results in the loss of virulence to the host plant rice. Two types of flagellins of *Pseudomonas aeruginosa* are also glycosylated. Archael (*Methanococcus voltae*) flagellins are highly glycosylated. The meaning of modification in the latter two cases is not clear.

Cap protein

Flagella have been regarded as a self-assembly systems. Indeed, flagellin can polymerize into flagella under conditions that commonly promote protein crystallization *in vitro*. However, *in vivo*, flagellin assembly requires another protein, without which the flagellin is secreted into the medium as monomers. The protein that helps filament formation is located at the tip and is thus called the cap protein or HAP2 or FliD.

The three-dimensional structure of the cap has been solved at the atomic level. The cap proteins assemble in a pentamer, forming a star-shaped structure. The star hands fit in the grooves of flagellin subunits at the tip of flagellum, leaving a small gap for a nascent flagellin to insert.

Hook

Shape

Hook, as the name suggests, is more sharply curved (almost right-angled) than filament and is much shorter. The curvature indicates the flexibility of the hook, though it has to be stiff enough to transmit torque generated at the basal structure to the filament. From these physical properties, the hook has been regarded as a universal flexible joint. The physical property of the hook is important in understanding the behavior of the tethered cells (see ‘Function’).

The length of the hook is 55 nm with a standard deviation of 6 nm, which is rather well controlled at constant when compared with the length of filament. However, this large standard deviation, 10% of the mean, indicates that the hook length control might be ‘loose’ or variable. At the moment, there are two models for the length control mechanism: the molecular ruler model and the measuring cup model. Polyhook is a mutant hook of indefinite length, obtained in *fliK* mutants. The former model claims that FliK measures the hook length, whereas the latter argues that the hook length is the results of polymerization of a defined number of hook subunits (see 'Morphological pathway').

It is not clear whether the polyhook is a tandem polymer of the wild-type hook. Its shape is a right-handed superhelix. The wild type hook has the same superhelix but consists of about one-fourth of the helical pitch.

Hook protein

The hook is a tubular polymer made of a single kind of protein: hook protein or FlgE. The molecular size of hook protein varies from 264 amino acids (*Bacillus subtilis*) to 718 amino acids (*H. pylori*), but is around 400 amino acids for most species.

The architecture of hook protein resembles that of flagellin: the amino acid sequence in both terminal regions is well conserved, but in the central region it is variable. Hook protein folding also completes on assembly. The three-dimensional structure of the hook protein in the hook has been solved at the atomic level.

Scaffolding protein FlgD

Hook does not self-assemble *in vivo*; it requires a helper protein, FlgD, which functions in a similar way as FliD does for filament formation. FlgD sits at the tip of the nascent hook to polymerize hook protein coming out from the central channel. When the hook length reaches 55 nm, FlgD is replaced by HAP1 (FlgK), which remains in the mature flagellum. Because of its temporary existence, FlgD is regarded as a scaffolding protein.

Hook-associated proteins

There are two minor proteins between the hook and filament. They are called hook-associated proteins (HAPs), because they were found at the tip of the hook in several filament-less mutants. Originally, there were thought to be three HAPs, called HAP1–3 in the order of their molecular size. HAP2 (FliD) turned out to be located at the tip of the filament as described above, leaving HAP1 (FlgK) and HAP3 (FlgL) between the hook and filament. They are, therefore, hook–filament junction proteins.

The number of subunits of HAP1 and HAP3 in a filament are estimated to be five or six, indicating that they form one-layer rings sitting one on another. The roles of these two HAPs have been ambiguous. The idea of a connector to smooth the junction between the two polymers is blurred by the question, 'why not one but two kinds necessary'. In a mutant of HAP3, filaments undergo polymorphic transitions so easily that cells cannot swim smoothly, suggesting a specific role of HAP3 as a stabilizer of filament structure.

Basal Structure

Flagella have to be anchored in the cell wall. The structural entity for the anchoring was called the basal structure or basal granule, hinted at by vague images by electron microscopy. Since DePamphilis and Adler in 1974 defined the details of the basal structure, it has been called the basal body. The basal body typically consists of four rings and one rod.

The basal body does not contain everything necessary for motor function. Fragile components were detached from the basal body during purification. In 1985, one such fragile structure was found attached to basal bodies purified by a modified method; it was named as the cytoplasmic ring (C ring). In 1990, another rod-like structure was found in the center of the C ring and named as the C rod. In 2006, flagellar export ATPase (FliI) was found at the periphery of the C ring as a complex with the supporter protein FliH. Therefore, the basal structure (as of 2007) consists of the basal body, the C ring, the C rod, and export ATPase, but there could be more.

Basal body

The basal body contains rings and a rod penetrating through them. The number of rings varies depending on the membrane systems: four rings in most of Gram-negatives and two rings in Gram-positives exemplified by *B. subtilis*. Some variations in the number (such as five rings in *C. crescentus*) have been occasionally seen. The fifth ring might be a ghost image of electron microscopy or could be erroneously added during ring formation (see 'Rod').

The structure of the basal body of *S. typhimurium* has been extensively analyzed. The physical and biochemical properties of the substructures of the basal body described below are from *S. typhimurium*, unless otherwise stated.

MS-ring complex

Earlier studies on flagellar motor function assumed that torque would be generated between the M and S rings, which face each other on the inner membrane. However in 1990, it was shown that a single kind of protein, FliF, self-assembles into a complex consisting of the M and S rings and a part of the rod.

FliF is 65 kDa, the largest of the flagellar proteins. It contains no cysteine residues. Overproduction of FliF in *Escherichia coli* gives rise to numerous MS-ring complexes packed in the inner membrane, indicating that the central channel is physically closed. The image analysis revealed that the MS-ring complex is composed of 26 subunits of FliF.

The S ring has been seen in the basal bodies from all the species studied so far (more than ten examples). It stays just above the inner membrane and has no apparent interaction with any other structures, thus named

S (supramembrane) ring. Besides, it is very thin and the role of the S ring remains mysterious.

Although the MS-ring complex is no longer regarded as the functional center of the flagellar motor, it is still the structural center of the basal structure and, as will be seen later (see 'Genetics'), plays an important role in flagellar assembly.

Rod

The rod is not as simple as its name suggests; it is structurally separated into two parts: the proximal rod and the distal rod of 10 nm length. The proximal rod consists of three proteins (FlgB, FlgC, and FlgF), while the distal rod consists of only one kind of protein FlgG. It breaks at the midpoint when external physical force is applied to the filament, which is usually not expected for the structure that transmits torque to the filament.

Rod formation seems complicated because of the four component proteins. No intermediate rod structure has been observed; either there is a whole rod or no rod at all. Several *flgG* point mutants produce distal rods *c.* 60 nm long. Double mutants of a *flgG* point mutation and *fliK* deletion give rise to polyrods, rods of undefined length, indicating that FliK controls the rod length by the same mechanism for the hook-length control. This also implies that the wild-type rod has a self-closed structure to keep the length as short as 10 nm. Since many P rings are formed on the long distal rod, FlgG is the target for interaction with FlgI, the subunit of the P ring (see below).

LP-ring complex

The outermost ring, the L ring, interacts with the lipopolysaccharide layer of the outer membrane, and the P ring just beneath the L ring may bind to the peptidoglycan layer. The LP-ring complex works as a bushing, fixed firmly enough to hold the entire flagellar structure stably in the cell surface.

The component proteins, FlgH for the L ring and FlgI for the P ring, have signal peptides, indicating that they are secreted through the general secretory pathway (GSP), which is the exception for flagellar proteins as will be seen later (see 'Genetics'). FlgH undergoes lipoyl modification.

P ring formation precedes L ring formation; without the P ring, L ring formation does not occur. Once the L and P rings have bound together to form the LP-ring complex, this complex is extraordinarily resistant against extremes of pH or temperature. Treatments with pH11, pH2, or boiling for a minute do not destroy the complex, confirming that the complex serves as a rigid bushing in the outer membrane. Essential roles of the complex are still ambiguous because of the facts that no corresponding structure has been found in Gram-positive bacteria and spirochaetes.

C ring

The C ring is a fragile component of the basal structure. It is resistant to the nonionic detergent Triton X-100 but is destroyed by the alkaline pH or high concentration of salts employed by the conventional purification method. The dome shape of the C ring is easily flattened on a grid during preparation for electron microscopy. Under the well-controlled conditions, the C ring shows a flat cup shape, whose structure is solved at 2-nm resolution by electron microscope image analysis.

The C ring consists of the switch proteins (FliG, FliM, and FliN) necessary for changing the rotational direction of the motor, and so is called the switch complex. FliG directly binds to the cytoplasmic surface of the M ring. FliM binds to FliG, and FliN to FliM. Stoichiometry of these molecules in the C ring is determined from the high-resolution image of the C ring: 24–26 copies of FliG, 32–36 copies of FliM and FliN. The copy number of FliN can be increased up to 100 without affecting the motor function.

Genetic studies revealed that the switch complex plays important roles in flagellar formation, torque generation, and switching of rotational direction. FliM in the C ring directly binds signal molecules, CheY, produced in the sensory transduction system, but the mechanism of the switching is still ambiguous.

Export apparatus

Flagella have been regarded as a self-assembly system, similar to that of bacteriophage. However, flagellar assembly is quite different from phage assembly in many ways. First, the flagellum, being an extracellular structure, assembles not in the cytoplasm but outside the cell. Second, therefore, the component proteins have to be transported from the cytoplasm to the outside. Third, consequently, assembly proceeds in a one-by-one manner at the distal end of the nascent structure.

For this kind of assembly, a protein secretion system must play an important role. As a matter of fact, among 14 genes required in the very first step of flagellar assembly, more than half of the gene products are necessary to form a protein complex called an export apparatus. One of them, FliI, has an ATPase activity, suggesting that one step in the export process requires ATP hydrolysis as an energy source.

The physical body of the export apparatus has not been identified yet; the C rod is a strong candidate, judging from its location within the C ring. Genetic analysis indicates that at least five components are required for the C rod: FliP, FliQ, FliR, FlhA, and FlhB, all of which have membrane-spanning region(s). How these component proteins are inserted in the small space of the central area of the MS-ring complex is still mysterious.

Function

The function of flagella is described here briefly so that the meaning of structure can be understood.

Bacterial flagella rotate. There is no correlation between bacterial flagella and eukaryotic flagella either in function or in structure; the type of movement, the energy source, and the number of component proteins differ greatly between the two. No evolutionary correlation between these two types of flagella has been shown.

Among motile bacterial species, swimming by flagellar rotation is the most common. However, several families such as Myxococcus, Mycoplasma, and Cyanobacteria can move on a solid surface by a gliding motion; myxococcus moves by means of type IV pili and mycoplasm has motor proteins as the locomotive organ. The motile organ of many other gliding bacteria is not known.

Torque

Rotational force (torque) of the flagellar motor is difficult to measure directly but can be estimated from the rotational speed of flagella. The method most widely employed is the tethered cell method, developed by Silverman and Simon in 1974. The rotation of a cell body caused by a tethered filament can be observed with an ordinary optical microscope. Rotating flagella on a cell can be observed by dark-field microscopy, developed independently by Robert M. Macnab and Hirokazu Hotani in 1976. Using laser as the light source of a dark-field microscope, the rotational speed of a flagellum on a cell stuck on the glass surface can be measured at the time resolution of milliseconds. A more sophisticated method employs the fluorescent microscope to detect the rotation of fluorescent beads attached to the hook. Most of the experiments for measuring torque have been done by Howard C. Berg and his colleagues.

Rotational direction

Flagella of many species (e.g., enterobacteria) can rotate in both the directions: clockwise (CW) and counterclockwise (CCW). Under ordinary circumstances, around 70% of the time is occupied by CCW rotation, which causes smooth swimming. A brief period of CW rotation causes a tumbling motion of the cell. There is no perceptible pause in switching between the two modes.

In some bacterial species such as *Rhodobacter sphaeroides*, a lateral flagellum on a cell rotates in the CW direction only with occasional pauses. During pauses, the filament takes on a coiled form and curls up near the cell surface. Upon application of torque on this filament, the coiled form extends to a semistable, right-handed form that closely resembles a curly form. The CW rotation of this right-handed helix causes a forward propulsive force on the cell.

Rotational speed

Flagella on a stuck cell rotate at *c.* 200 Hz, which is comparable with that of a free-swimming cell. In contrast, tethered cells rotate only at *c.* 20 Hz. It has been long believed that the cell body is too large and thus hydrodynamically too heavy for a tiny motor to rotate faster. However in 2007, we have shown that tethered cells almost always interact with the surface; the distance between the cell and the glass surface is only 55 nm, the hook length. The hook seems to play an active role as a flexible joint in the interaction.

The rotational speeds of flagella correlate directly with the torque and inversely with the viscosity of the solution. The correlation appears as a straight line in a speed–torque diagram – the higher the viscosity, the slower the speed is. This indicates that the torque of the flagellar motor is constant over a wide range of speed.

The highest speed so far measured is 1700 Hz for *V. alginolyticus*. However, only 10% of the torque derived from the speed is used as a propulsive force, the rest being lost in slippage of flagella in the medium. In general, cells with single polar flagellum swim ($>100\,\mu\mathrm{m\,s^{-1}}$) faster than cells with peritrichous flagella ($<20\,\mu\mathrm{m\,s^{-1}}$).

Energy Source

The energy source of torque generation in the flagellar motor is not ATP but proton motive force (PMF). PMF is the electrochemical potential of the proton and results in the flow of protons from outside to inside the cell. PMF consists of two different forms of energy: membrane potential, and entropy caused by a difference in pH between outside and inside the cell. Since these two parameters are independent and separable from each other, either one can, in principle, be abolished without affecting the other. It is not known whether protons directly flow through the flagellar motor to generate torque or flow nearby to induce conformational changes of the component proteins in the C ring.

One of the goals of flagellar research is the elucidation of the mechanism by which PMF is converted into torque in the motor.

Switching of Rotational Direction

Switching of the rotational direction of flagella is the primary basis of chemotaxis, one of the most important behaviors shown by bacteria. Damage in the switching mechanism results in a rotation biased to either CCW only or CW only. Although cells that rotate only CCW swim smoothly in liquid, they cannot form a chemotaxis ring on a semisolid agar plate.

In a strict sense, the switching mechanism will not be solved until the mechanism of rotation is solved. However, factors involved in the mechanism are known;

an effector binds to FliM of the switch complex in the flagellar motor. The effector is the phosphorylated form of CheY, a signaling protein within the sensory transduction system.

Genetics

Flagellar genetics has been most extensively studied in *S. typhimurium*, especially using the enormous number of SJW (Salmonella Japan Waseda) strains that Shigeru Yamaguchi has collected for more than 30 years. Once the fundamental genetics was established, molecular biology has been serving powerful tools to reveal the details. Topics regarding the *fla* genes in this section are mostly based on results obtained from *Salmonella* strains, whereas chemotaxis (*che*) genes and motility (*mot*) genes were more extensively analyzed using mostly *E. coli* and other strains (e.g., *Rhizobium* spp., *Vibrio* spp., etc.).

Flagellar Genes

There are more than 50 flagellar genes divided into three types according to null mutant phenotype (**Figure 3**).

The fla *genes*

Defects in the majority of the flagellar genes result in flagellar-deficient (Fla-) mutants. These genes were originally called *fla* genes. In 1985 when the number of genes exceeded the number of letters in the alphabet, a unified name system for both *E. coli* and *S. typhimurium* was proposed by Robert M. Macnab and introduced in the field with approval by all contemporary geneticists. They are *flg*, *flh*, *fli*, and *flj*; each for one of the clusters of genes scattered in several regions around the chromosome (see 'The mot genes'). When a particular gene does not have the corresponding gene in *Salmonella*, historical names are kept, for example, *flaF*, *flaG*, *flbS*, and *flbT*.

The mot *genes*

Mutants that produce paralyzed flagella are called motility-deficient (Mot-) mutants. There are only two *mot* genes (*motA* and *motB*) in *S. typhimurium*. In *V. alginolyticus*, there are two sets of flagella – polar and lateral. The energy source for each motor is distinguishable; the sodium motive force for the polar flagellum and the PMF for the lateral flagella. *Mot* genes for the polar flagellum are called *pomA* and *pomB*. There are four more *mot* genes (*motA, motB, motX, and motY*) in *V. alginolyticus*, *R. sphaeroides*, and *Aeromonas hydrophila*. *V. alginolyticus* MotX and MotY are associated with the basal body of sodium-driven polar flagellum and required for stator formation.

P. aeruginosa retains one set of *mot* genes (*motA* and *motB*) and another set (*motC* and *motD*). Although it is indicated that the *motC* and *motD* genes play an important role in pathogenicity, their roles in motor function are not known. There was renaming of some mot genes; MotD of *Sinorhizobium meliloti* and related alpha-proteobacteria turned out to be the flagellar-hook-length regulator FliK, indicating that original naming was wrong. MotE in *S. meliloti* is a chaperone specific for the periplasmic motility protein, MotC.

The che *genes*

Mutants that can produce functional flagella but cannot show a normal chemotactic behavior are called

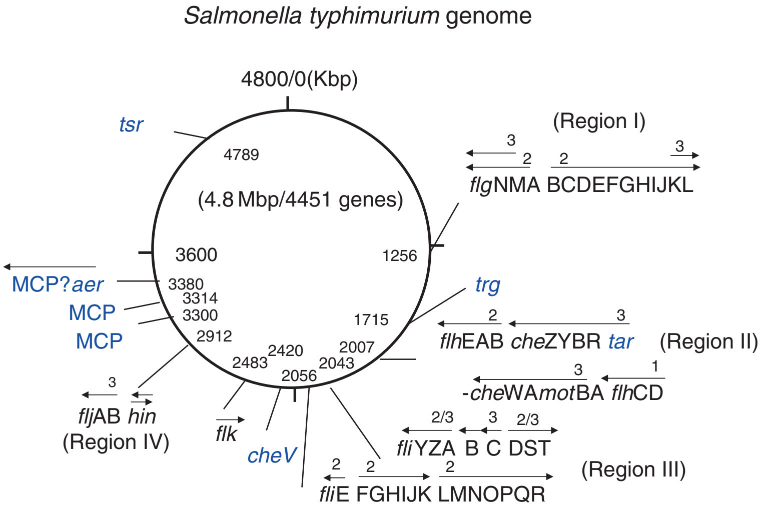

Figure 3 Genetic map of *Salmonella typhimurium*. Flagellar genes distribute in several clusters on the chromosome. Arrows over genes indicate the size of operons and their transcriptional directions. The numbers on the arrows indicate classes of transcription. The regulation of class 2 and 3 is not simple; some operons are expressed twice in class 2 and class 3. Chemotaxis receptors (Tsr, Trg, and Tag) and receptor homologues (MCP) are scattered on the chromosome.

chemotaxis-deficient (Che-) mutants. These are divided into two types – general chemotaxis mutants (authentic Che-) and specific chemotaxis mutants. The former involve the proteins working in the sensory transduction (CheA, CheW, CheY, CheZ, CheB, and CheR), and the latter involve the receptor proteins (e.g., Tsr, Tar, Trg, and Tap).

In some species, there are multiple homologues of the *E. coli* chemotaxis genes. For example, *R. sphaeroides* possesses 5 × *cheA*, 2 × *cheB*, 2 × *cheR*, 3 × *cheW*, and 6 × *cheY* arranged in several operons. Thirteen chemoreceptors, including both membrane-spanning and cytoplasmic or transducer-like proteins (Tlps), have been identified (as of 2007). These are differentially expressed according to the environmental conditions. It is not known whether the products of the *che* operons operate through independent, linear pathways or there is significant cross-talk between the components of these operons.

In *V. cholerae*, there are five *cheY* genes including one putative *cheY*. It was shown that only one of them directly switches flagellar rotation. In *E. coli*, CheY dephosphorylation by CheZ extinguishes the switching signal. But instead of CheZ, many chemotactic bacteria contain CheC, CheX, and FliY for dephosphorylation.

Gene Clusters in Four Regions

Most flagellar genes are found in gene clusters on the chromosome. They are in four regions: the *flg* genes in region I, the *flh* genes and *mot* and *che* genes in region II, and the *fli* genes in regions IIIa and IIIb (**Figure 3**). The *flj* operon (including *fljA* and *fljB*) in region IV involves an alternative flagellin gene to *fliC* and is only found in *Salmonella*. Either FliC flagellin or FljB flagellin is produced at one time. The *hin* gene inverts the transcriptional direction at a certain statistical frequency; if the *flj* operon is being expressed, FljA represses *fliC*, allowing FljB flagellin alone to be produced. This alternate expression of two flagellin genes is called 'phase variation'.

This clustering of flagellar genes is also observed in other peritrichously flagellated species; grouping of particular genes and gene order are similar to those in *S. typhimurium*. Flagellar genes for the polar flagellum in *Pseudomonas* spp. and *Vibrio* spp. form clusters in a few regions, but the gene order is different from that of peritrichous flagella. The polar flagellum requires special regulatory genes (such as *fleP, fleQ, flhF, flhG*) to localize one flagellum at the pole of the cell, which peritrichous flagella do not require. Flagellar genes of *C. crescentus* or *Campylobacter jejuni* are scattered in more than seven regions; the extreme case is *H. pylori*, in which single flagellar genes or at most three-gene clusters are scattered all over the chromosome. It is interesting to see that a cluster, FliC (flagellin)–FliD (the capping protein)–FliS (chaperone for FliC)–FliT (chaperone for FliD), is ubiquitously found in all species but not in *H. pylori* and *Buchnera* spp.

Transcriptional Regulation

Flagellar construction requires a well-ordered expression of flagellar genes not only because there are so many genes, but also because flagellar assembly requires only one kind of component protein at a time, as described in previous sections. There is a strict hierarchy of expression among the flagellar genes. The hierarchy is controlled or maintained by a few prominent regulatory proteins.

Hierarchy: Three classes

The hierarchy of flagellar gene expression is divided into three classes: class 1 regulates class 2 gene expression, and class 2 regulates class 3. Class 1 contains the master genes: only two genes in one operon, *flhD* and *flhC*.

Class 2 consists of 35 genes in eight operons. There are two regulatory genes, *fliA* and *flgM*; the rest are component proteins of the flagellum or the export apparatus.

Class 3 genes code flagellin, MotA and MotB, and all the Che proteins. Flagellin is one of the most abundant proteins in a cell, suggesting that the tight regulation in the hierarchy guarantees the economy of the cell.

There is another class of regulation pathway in species that produce polar flagellum. In *P. aeruginosa*, there are four classes; class 1 contains the master gene *fleQ*, class 2 contains 25 genes necessary for constructing the export apparatus, class 3 contains 12 genes necessary for completing the hook-basal body, and class 4 contains 13 genes to produce the filament and the chemotaxis system.

Master genes, flhDC

Master gene products form a tetrameric complex of FlhD/FlhC, which works as a transcriptional activator of the class 2 operons.

The master operon (*flhDC*) is probably transcribed with the help of the 'housekeeping' sigma factor, σ^{70}. The master operon is also activated by a complex of cyclic AMP and catabolite activator protein (cAMP–CAP), which binds to a site upstream of the promoter.

In *P. aeruginosa*, *fleQ* is the master gene instead of *flhCD*. However, *fliA* is out of the control of *fleQ*; the controlling element of *fliA* is not known yet.

Sigma factor F (σ^F: FliA) and antisigma factor (FlgM)

The FliA and FlgM proteins expressed from the operon competitively regulate the class 3 operons. FliA is the sigma factor that enhances the expression of the class 3 operons, while FlgM is an antisigma factor against FliA.

If the hook and basal body have been constructed normally, FlgM is secreted into the medium through the basal body and the complete hook, allowing free FliA proteins to work on the class 3 operons. However, if the hook and basal body construction is somehow halted in the middle of process, FlgM stays in the cytoplasm in a complex with FliA, maintaining shut-off of the expression of the class 3 operons.

A combination of *fliA–flgM* genes has been found in genomes of all species so far studied except *Buchnera* spp. that lacks all of the *fla* genes necessary for filament formation.

Global regulation versus internal regulation

There are several external genes or factors that affect the flagellar gene expression through the master operon *flhDC*. Some of the factors show pleiotropic effects on many cellular events such as cell division, suggesting that flagellation would be finely tuned with the cell division cycle due to well-organized tasks of global regulation systems.

As described above, the master operon (*flhDC*) is probably transcribed using the 'housekeeping' sigma factor, σ^{70}. In the last decades, other factors regulating or modulating *flhDC* expression have been identified, mainly in *E. coli*. The motility of *E. coli* cells is lost at temperatures higher than 40 °C as a result of reduced *flhDC* expression. It has been shown that some of the heat-shock proteins are involved in both class 1 and class 2 gene expressions. This strongly suggests that flagellar genes are under global regulation in which the heat-shock proteins play a major role; probably the proper protein folding (or assembly) mediated by these chaperons is essential for flagellar construction.

Other adverse conditions such as high concentrations of salts, carbohydrates, or low-molecular-weight alcohols also suppress *flhDC* expression, resulting in lack of flagella. The regulation by all these factors is independent of the cAMP–CAP pathway.

Flagellar gene expression is also controlled by the ubiquitous bacterial second messenger cyclic diguanosine monophosphate (c-*di*-GMP). In response to changing environments, fluctuating levels of c-*di*-GMP inversely regulate flagellar formation and thus cell motility.

The factors and mechanism of directly turning on the master *flhDC* operon in accordance with the cell cycle are yet unknown. Another goal of the flagella research is the elucidation of the roles of global regulators on flagellar gene expression to uncover the complex regulatory network connecting flagellation and cell division.

Morphological Pathway

The order of the steps toward the construction of a flagellum (the morphological pathway) has been analyzed in the same way as was used for bacteriophage – analyzing intermediate structures in various flagellar mutants and aligning them in size from small to large ones. Flagellar construction starts from the cytoplasm, progresses through the periplasmic space, and finally extends to the outside of the cell (**Figure 4**).

In the cytoplasm

The smallest flagellar structure recognizable by electron microscopy is the MS-ring complex; therefore, the MS-ring complex is regarded as the construction base. When two other flagellar substructures, the C ring and the C rod, attach on the cytoplasmic side of the M ring, the gigantic complex starts secreting other flagellar proteins to construct the flagellum.

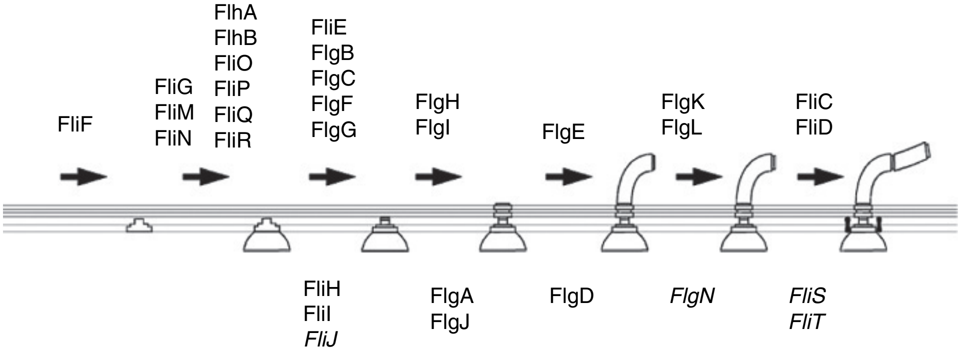

Figure 4 Morphological pathway of flagellation. Flagellar construction proceeds from left to right. Gene products shown above the membranes are incorporated in the flagellar structure at each step. The gene products shown under the membranes are chaperones for the component protein just above the membrane (shown in italics) or enzymes: FliI is an ATPase, and FlgJ is muramidase.

In the periplasmic space

The first extracellular structure constructed on the MS-ring complex is a rod. When the rod has grown large enough to reach the outer membrane, the hook starts growing. However, the outer membrane physically hampers the hook growth until the outer-ring complex makes a hole in it. Among flagellar proteins, FlgH and FlgI, the component proteins of the outer-ring complex, are exceptional in terms of the manner of secretion; these two proteins have cleaved signal peptides and are exported through the GSP. However under special conditions, filaments grow in the absence of the outer rings but stay in the periplasm to form the periplasmic flagella as seen in spirochaetes.

Outside the cell

Once the physical block by the outer membrane has been removed, the hook resumes growth with the aid of FlgD until the length reaches 55 nm. Then, FlgD is replaced by HAPs, which is followed by the filament growth. The filament growth proceeds only in the presence of FliD (HAP2 or filament cap protein); without this cap, exported flagellin molecules are lost to the medium.

Flagellar Protein Export as a Type III Secretion System

There are several ways to transport proteins outside bacterial cells. The best-known pathway is the GSP. However, many flagellar proteins cannot pass through this system, since they do not have the signal sequences that are necessary for the recognition by GSP.

There are six characterized bacterial protein secretion systems (type I–VI) that are grouped according to their function in pathogenesis. Here I will briefly explain the major three types of export systems.

Type I secretion system (T1SS) secretes proteins without modification through the secretion apparatus consisting of a few component proteins that span both the inner and the outer membranes, for example, hemolysin in *E. coli*.

Type II secretion system (T2SS) secretes proteins retaining the signal peptide, which is cleaved upon secretion by GSP, for example, pullulanase in *Klebsiella oxytoca*. Thus, GSP is a secretion machinery in T2SS.

Type III secretion system (T3SS) secretes proteins without cleavage through the gigantic secretion apparatus spanning both the inner and the outer membranes, for example, virulence factors from many pathogenic *Enterobacter* spp. The flagellar protein export system is now regarded as a T3SS. The flagellar export apparatus consists of at least six components (FlhA, FlhB, FliI, FliP, FliQ, and FliR). The amino acid sequences of these proteins share homology with those for secretion of virulence factors in many pathogenic bacteria. The structures between these two distinguishable systems resemble each other; the secretion apparatus for virulence factors is the needle complex, which looks like the flagellar basal body, consisting of several ring structures and a needle. It is now suspected that flagellum and pathogenesis might be derived from a common ancestor.

The two secretion systems are superficially independent from each other in *S. typhimurium.* However, it is now known that the *Salmonella* pathogenesis Island (SPI) 1 gene expression is regulated by *fliZ* in serovar Typhimurium and is dependent on flagellar sigma factor FliA in serovar Typhi. Note that *fliA* and *fliZ* sit next to each other in an operon.

The Kinetics of Morphogenesis

The morphological pathway of the flagellum described above indicates the order of the construction steps but ignores the time to be consumed at each step. In order to achieve coherent cell activities, flagellar construction has to be synchronized with cell division. The most time-consuming step of flagellation seems to be the first step, the construction of the export apparatus, which takes almost one generation to complete. The filament elongation also takes time; filaments grow over generations.

The growth processes of the filament and the hook have been carefully analyzed. By taking a closer look at elongation modes of these two polymers, we will get a glimpse of the whole kinetic process of flagellar construction.

Filament growth

In bacteria with peritrichous flagella, the number and the length of flagella are, if not exactly, fairly well defined; there are 7–10 flagella per cell and the average length of filament is 5–8 μm.

A defined number of flagella have to be supplied at each cell division. A large deviation from this number will cause disastrous results to the cell – either no flagella at all or too many to swim. The number of flagella must be genetically controlled, but the gene(s) for this role has not been found.

On the other hand, filament growth seems free from genetic control since it continues over generations. From statistical analysis of the length distribution, the elongation rate of filaments is inversely proportional to the length; thus, a filament grows rapidly in the beginning and gradually slows down to a negligible rate.

Hook growth

In contrast to the wide distribution of filament lengths, the hook length is rather well controlled at 55 nm with a deviation of 6 nm. The hook length is controlled by a secreted protein FliK; deletion of the *fliK* gene results in hooks with undefined length, called polyhooks. Engineered FliK, either elongated by insertion of foreign sequences or shortened by internal deletions, gives rise to hooks whose length is proportional to the molecular size of the mutant FliK. Thus, FliK is a molecular ruler. But it must work in the cytoplasm because some mutant FliK control the hook length but are not secreted. In 2001, we have shown that mutations in switch proteins (FliG, FliM, and FliN) gave rise to short hooks with a defined length, indicating that the C ring serves as a measuring cup for hook monomers. The relationship between FliK and the C ring cup is not clear.

Statistical analysis of the length distribution of polyhooks reveals that the hook grows in a similar manner as the filament does; it starts outgrowing at 40 nm min^{-1} and exponentially slows down to reach a length of 55 nm. After the length is 55 nm, the hook grows at a constant rate of 8 nm min^{-1}. It takes many generations for polyhooks to grow as long as several micrometers.

Studies of the correlation between flagellation and cell division are underway, but no definite schemes have been found.

Conclusion

In the previous edition, I wrote, "Analysis of the flagellar structure has been coming to an end"; but since then many unexpected facts were revealed. We are proud of the brilliant results of our research; most components of the flagellum have been identified, the pathway of flagellar construction has been revealed, and roles of *ca.* 40 flagellar genes in the flagellar construction are now known. However, I now realize that the nature is not so shallow to reveal everything in a short life of a man. We have to keep asking 'Why?' with indefatigable enthusiasm to unveil mysteries of the flagellum.

We have not yet known the physics principle of flagellar rotation. One of the immediate goals is to answer a simple but important question; what is rotating against what? This question stems from the controversy that has started from the beginning of the flagellar research. Without knowing the rotor and the stator in detail, the mechanism of motor function will never be understood.

And then, we want to answer a more intriguing and difficult question: what is the ancestor of the flagellum? The question arose from the recent discovery of similarity between the flagellum and the pathogenicity: not only the gene sequences between the two distinguished systems are homologous, but also their supramolecular structures resemble each other. This also leads us to the most primitive question: what is the flagellum?

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Relevant Website

http://www-micro.msb.le.ac.uk/ – Microbiology @ Leicester

Forensic Microbiology

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S Morse, Centers for Disease Control and Prevention, Atlanta, GA, USA

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Biocrime Investigations
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Glossary

attribution Attribution is the information obtained regarding the identification or source of a material (to the degree that it can be ascertained).

biocrime or bioterrorism Biocrime or bioterrorism is the threat or use of microorganisms, toxins, pests, prions, or their associated ancillary products to commit acts of crime or terror.

bioweapon Bioweapon is any weapon comprised of a microorganism or toxic microbiological products to commit a crime or terrorist attack with the intent to cause harm to, death of, or disruption of humans, animals, or plants.

epidemiology Epidemiology classically studies the health and illness of a population; but herein is defined as the study of the combination of clinical presentation of disease, identification of the pathogen, the distribution of the pathogen in a population, and other factors to deduce where an infection began and how it spread throughout a population.

forensic science Forensic science generally is the application of science in the investigation of legal matters. Scientific knowledge and technology are used to serve as witnesses in both civil and criminal matters.

microbial forensics Microbial forensics is a scientific discipline that examines microorganisms, toxins, pests, prions, or their associated ancillary products for source attribution.

Select Agents Select Agents are biological agents and toxins that have the potential to present a serious threat to public, animal or plant health, or to animal or plant products. Lists of these agents have been defined by HHS and USDA. Registration is required for use or transfer of these agents.

signature Signature is a specific analytically derived characteristic that contributes to attribution.

Abbreviations

AFM	atomic force microscopy
AMS	accelerator mass spectrometry
BAMS	bio-aerosol mass spectrometry
CDC	Centers for Disease Control and Prevention
CSF	cerebrospinal fluid
DHS	Department of Homeland Security
EDX	energy dispersive X-ray microanalysis
FBI	Federal Bureau of Investigation
FMDV	foot-and-mouth disease virus
HEAT	Hazardous Evidence Analysis Team
INDELS	insertions/deletions
LC/MS	liquid chromatography/mass spectrometry
LRN	Laboratory Response Network
MALDI-TOF	matrix assisted laser desorption/ionization time of flight
MLST	multilocus sequence typing
NBACC	National Biodefense Analysis and Countermeasures Center
NBFAC	National Bioforensics Analysis Center
NIBC	National Interagency Biodefense Campus
PCR	polymerase chain reaction
PIXE	particle (proton)-induced X-ray emission

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RFLP	restriction fragment length polymorphism
SEM	scanning electron microscopy
SERS	surface-enhanced Raman spectroscopy
SNP	single nucleotide polymorphism
STIM	scanning transmission ion microscopy
ToF-SIMS	time-of-flight secondary ion mass spectrometry
USDA	United States Department of Agriculture
VEE	Venezuelan equine encephalitis virus
VNTR	variable number tandem repeat
WHO	World Health Organization

Defining Statement

Microbial forensics is a continually evolving discipline that examines microorganisms, toxins, pests, prions, or their ancillary products for source attribution. Challenges to developing a robust microbial forensics program involve issues of evidence integrity, extraction of trace biological signatures and data interpretation. The field continues to mature as it faces these challenges to the prevention and investigation of acts of bioterrorism.

Introduction

On 2 October 2001, a 63-year-old male photo editor working for American Media in Boca Raton, Florida, appeared in the emergency room complaining of fever, emesis, and confusion. A Gram stain of his cerebrospinal fluid (CSF) revealed the presence of numerous polymorphonuclear leukocytes and chains of large Gram-positive bacilli. *Bacillus anthracis* was isolated from the CSF after 7 h of incubation and from blood cultures within 24 h of incubation. On 4th October, the Florida Department of Health notified the public that a case of inhalational anthrax had been confirmed. Prior to this diagnosis, the last reported case of inhalational anthrax in the United States was in 1976, when a home craftsman died after using imported yarn contaminated with endospores of *B. anthracis*. The proximity of this case of inhalational anthrax to the 11 September 2001 terrorist attacks in New York City, Washington, DC, and Pennsylvania led to the speculation that this event might have a more sinister origin. The discovery of *B. anthracis* spores on the computer keyboard of the index case, a computer in the America Media building, and recovery of spores from asymptomatic coworkers and a hospitalized coworker supported the premise that the introduction of the spores was an act of terrorism. Letters containing *B. anthracis* spores were discovered, and a subsequent investigation resulted in a reconstruction of their routes (known and presumed) through the US mail system (**Figure 1**). Use of the US Postal Service to send spore-laden letters to media outlets (ABC, CBS, NBC, and the New York Post) and Congressional offices (Senators Tom Daschle and Patrick Leahy) led to widespread panic, and to one of the largest investigations ever undertaken by the Federal Bureau of Investigation (FBI). Law enforcement was ill-prepared to address these events from a forensic perspective; not in recent times had such a pernicious biological pathogen been used to perpetrate an attack on domestic soil. There was suddenly an urgent need for forensic methods that could be used to gather and characterize microbial evidence for attribution of acts of terrorism.

The mailing of *B. anthracis* spores brought biocrimes and acts of biological terrorism to the forefront of public attention. Biocrimes are, in actuality, assault crimes in which the weapon is a biological toxin or pathogen. Although acts of bioterrorism are also biocrimes, bioterrorism is generally motivated by ideological objectives (e.g., political, religious, ecological) and induces psychological fear and panic, which are inherent elements of terrorism. As such, just the threat of the use of a biological agent can constitute an act of bioterrorism. Biocrimes generally use known infectious agents that have proven capable of producing disease outbreaks among human and agricultural populations.

The threat that a microorganism or its toxin will be used as a weapon in a criminal act is greater today than ever before. A number of bacteria, viruses, and fungi (and their byproducts), which present serious health concerns to humans, livestock, and plants, could potentially be used as biological weapons. While not all toxins and pathogenic organisms would make useful weapons, a number of them could be used effectively. Many infectious and harmful microorganisms can be grown *in vitro* from a single cell with relative ease. Dispersal may not require sophisticated technology, particularly for agricultural and food targets. Criteria often considered important for assessing whether a microorganism or toxin may constitute a potential weapon are

1. accessibility;
2. culturability;
3. ease of large-scale production;
4. infectivity and toxicity;
5. consistency of death or disability;
6. delivery potential;

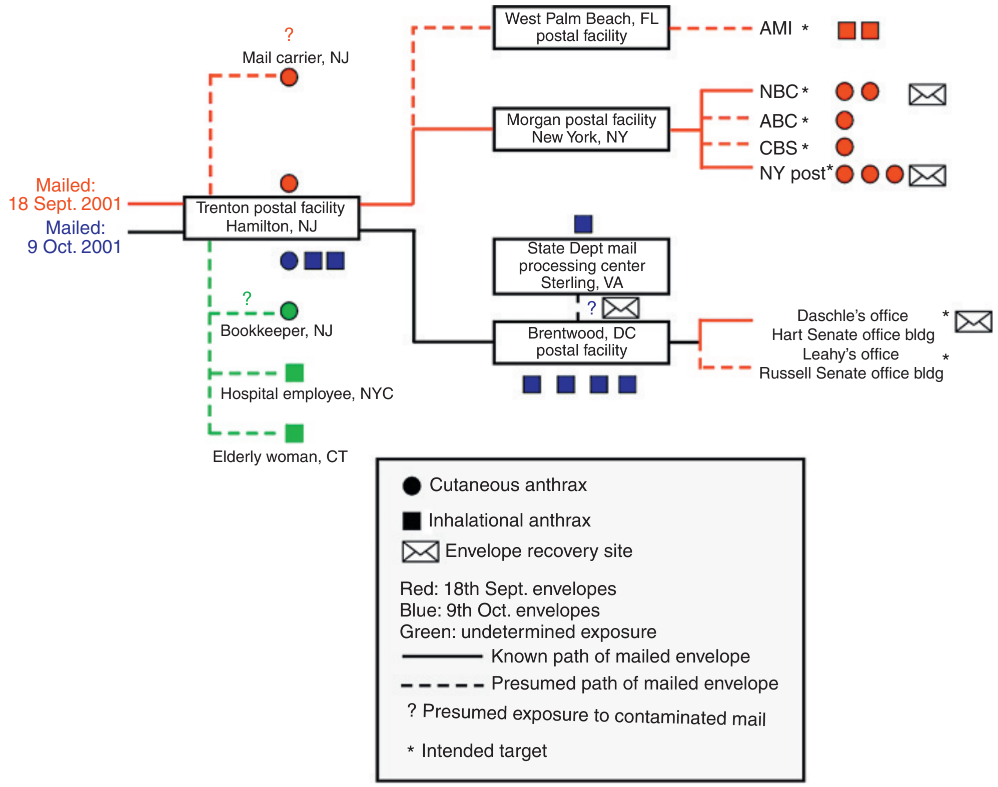
?
Mail carrier, NJ
Mailed:
18 Sept. 2001
Mailed:
9 Oct. 2001
Trenton postal facility
Hamilton, NJ
?
Bookkeeper, NJ
Hospital employee, NYC
Elderly woman, CT
West Palm Beach, FL
postal facility
AMI *
Morgan postal facility
New York, NY
NBC *
ABC *
CBS *
NY post*
State Dept mail
processing center
Sterling, VA
?
Brentwood, DC
postal facility
Daschle's office
Hart Senate office bldg *
Leahy's office
Russell Senate office bldg *
● Cutaneous anthrax
■ Inhalational anthrax
✉ Envelope recovery site
Red: 18th Sept. envelopes
Blue: 9th Oct. envelopes
Green: undetermined exposure
Known path of mailed envelope
Presumed path of mailed envelope
? Presumed exposure to contaminated mail
* Intended target

Figure 1 Reconstruction of the paths of anthrax-tainted letters through the US Postal system and the associated disease outbreak. ABC, American Broadcasting Company; AMI, American Media, Inc.; CBS, Columbia Broadcasting System; NBC, National Broadcasting Company. Adapted from Jernigan JA, Stephens DS, Ashford DA, *et al.* (2001) Anthrax bioterrorism investigation team. Bioterrorism-related inhalational anthrax: The first 10 cases reported in the United States. *Emerging Infectious Diseases* 7: 933–944.

7. stability and retention of potency during transport and storage;
8. potency after dissemination; and
9. economic effects (e.g., foreign animal diseases).

A combination of lethality, availability, and deliverability must be present before a pathogen or toxin can be used as an effective weapon, although a hoax may not require any of these criteria for the material used. For example, the need for lethality depends on the purpose. Foot-and-mouth disease virus (FMDV) is not a lethal infection, but one case can have a disastrous effect on the economy.

Infectious diseases account for 29 of the 96 major causes of human mortality and morbidity and for approximately 25% of global deaths (i.e., 14 million) per year. Many of the responsible pathogens exist in nature; thus, traditional security measures alone will not suffice to prevent access. However, the Select Agent Regulation was promulgated to limit access to the most harmful or plausible pathogens. It regulates the possession and transfer of particularly harmful microorganisms (and in some cases nucleic acids) and toxins. The Centers for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA) are responsible for enforcing this regulation and have compiled lists of microorganisms and toxins (i.e., Select Agents) that are deemed to pose a significant threat to the health of humans, livestock, and plants (**Tables 1** and **2**).

Responding to the intentional release of biological agents that affect humans is the role of public health. To facilitate planning efforts that involve the stockpiling of antibiotics or the development of diagnostic assays and vaccines, the CDC developed a list of priority threat agents (**Table 1**). This list was divided into three groups referred to as Category A, B, and C agents. Category A agents are those most likely to cause mass fatalities; Category B agents cause moderate rates of illness with lower death rates; and, Category C agents are responsible for a number of emerging infectious diseases. These agents were considered representative of the immediate needs for public health preparedness efforts; however, genetic engineering and synthetic technology may broaden the threat list in the future. Furthermore, this list is periodically revised by the Department of Homeland Security (DHS) to reflect changes in preparedness levels and current threat assessments.

The potential impact of an attack with a biological agent was estimated in a World Health Organization (WHO) study in which hypothetical biological attacks with three

Table 1 CDC high-consequence pathogens and toxins

Category A	*Category B*	*Category C*
Bacillus anthracis (anthrax)	Alphaviruses (Venezuelan equine encephalitis)	Hanta virus
Clostridium botulinum toxin	*Brucella* spp.	*Mycobacterium tuberculosis* (multidrug resistant tuberculosis)
Francisella tularensis (tularemia)	*Burkholderia mallei* (glanders)	Nipah virus
Hemorrhagic arenaviruses (Lassa fever)	*Burkholderia pseudomallei* (Melioidosis)	Tick-borne encephalitis
Hemorrhagic fever filoviruses (Ebola)	*Chlamydia psittaci* (psittacosis)	Tick-borne hemorrhagic fever viruses
Variola major (smallpox)	*Clostridium perfringens* (epsilon toxin)	Yellow fever
Yersinia pestis (plague)	*Coxiella burnetti* (Q fever)	
	Cryptosporidium parvum (cryptosporidiosis)	
	Escherichia coli O157:H7	
	Ricin toxin (castor bean – *Ricinus communis* – extract)	
	Rickettsia prowazekii (Typhus)	
	Salmonella spp.	
	Shigella spp.	
	Staphylococcus enterotoxin	

viral (Rift Valley fever, Tick-borne encephalitis, and Venezuelan equine encephalitis) and two bacterial pathogens (*Francisella tularensis* and *B. anthracis*) were modeled. The results (**Table 3**) demonstrated that an attack with *B. anthracis* or *F. tularensis* would result in more deaths and casualties than an attack with any one of the viral agents, with the caveats that it required 50 kg of the agent and optimal wind conditions to produce these results. In this scenario, almost half of the exposed population would be killed or incapacitated by the release of *B. anthracis*. There have been changes in the human population in the 30 years since this model was presented. The most important could be the increase in the incidence of immuno-compromised individuals. Their weakened immune systems could result in a higher risk of infectivity and passage of the pathogen than was previously calculated. However, more simulations and risk assessment studies are needed to present a current view of the impact of a release of a biological pathogen into the population.

Law Enforcement/Forensic Role

Law enforcement has the responsibility of protecting individuals and communities against bioterror threats. These responsibilities include deterrence, interdiction, response to, and investigation of such criminal acts. The forensic sciences can assist in these investigations by providing additional leads that may attribute the source of an evidentiary sample and/or assist in identifying the perpetrator. The field of microbial forensics was created in response to the threat of bioterrorism and is a discipline dedicated to the characterization, analysis, and interpretation of evidence from the scene of an act of bioterrorism or a biocrime. Microbial forensic investigations are carried out to obtain information regarding the identification or source of the material (i.e., attribution) used in a bioterrorism act, biocrime, hoax, or unintentional release, with the ultimate goal of identifying those responsible (as well as excluding those not associated with the crime). In order to perform these functions with best practices and by employing the best analytical tools available, the field of microbial forensics is continually evolving. At a minimum, microbial forensics incorporates the fields of microbiology, molecular biology, genetics, biochemistry, genomics, epidemiology, and bioinformatics. Many of the laboratory techniques in use or under development for use in the discipline have been reviewed elsewhere. This article will present the challenges in this new field of forensic investigation and the domestic network that has been established to respond to and investigate incidents involving the use of biological agents. For a history of the use of biological weapons, the reader is referred to recent reviews.

Microbial Forensics Response

An intentional attack with a biological agent can be classified as either overt or covert. An overt attack is often recognized immediately (e.g., anthrax-laden letters received in the Hart Senate Office Building), while a covert attack may not become known for days, weeks, or years, if at all. The ability to distinguish naturally occurring outbreaks from a covert dissemination of a biological agent can be a challenge for epidemiologists, as exemplified by the investigation in The Dalles, Oregon, of a food-borne outbreak of *Salmonella typhimurium* in a salad bar. The possibility of intentional contamination was considered early in the investigation, but was excluded for the following reasons:

Table 2 USDA high consequence pathogens and toxins

Bacillus anthracis (anthrax)
Brucella spp.
Burkholderia mallei (glanders)
Burkholderia pseudomallei (Melioidosis)
Cowdria ruminantium (heartwater or cowdriosis)
Coxiella burnetti (Q fever)
Francisella tularensis (tularemia)
Mycoplasma capricolum (contagious caprine pleuropneumonia)
Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)
Coccidioides immitis (true systemic (endemic) mycoses)
Akabane virus
African horse sickness virus
African swine fever virus
Avian influenza virus
Blue tongue virus
Camel pox virus
Classical swine fever virus
Eastern equine encephalitis virus
Foot-and-mouth disease virus
Goat pox virus
Hendra virus
Japanese encephalitis virus
Lumpy skin disease virus
Malignant catarrhal fever virus
Menangle virus
Newcastle disease virus
Nipah virus
Peste des petits ruminants virus
Rift Valley fever virus
Rinderpest virus
Sheep pox virus
Swine vesicular disease virus
Venezuelan equine encephalitis virus
Vesicular stomatitis virus
Bovine spongiform encephalopathy agent
Clostridium botulinum toxin
Clostridium perfringens epsilon toxin
Shigatoxin
Staphylococcus enterotoxin
T-2 toxin

Table 3 Casualty estimates produced by hypothetical biological attack

	Downwind		
Agent	*Reach (km)*	*Dead*	*Incapacitated*
Rift Valley fever	1	400	35 000
Tick-borne encephalitis	1	9500	35 000
Venezuelan equine encephalitis virus (VEE)	1	200	19 800
Francisella tularensis	>20	30 000	125 000
Bacillus anthracis	>20	95 000	125 000

These estimates are based on the following scenario: release of 50 kg of agent by aircraft along a 2 km line upwind of a population center of 500 000. Reproduced from Meyer RF and Morse SA (2007) Bioterrorism. In: Mahy B and van Regenmortel M (eds.) *Encyclopedia of Virology*, 3rd edn. Oxford: Elsevier.

(1) such an event had never been reported previously; (2) no one claimed responsibility; (3) no disgruntled employee could be identified; (4) there was no apparent motive; (5) the epidemic curve suggested multiple exposures, which was presumed to be unlikely behavior for a saboteur; (6) law enforcement officials failed to establish a recognizable pattern of unusual behavior; (7) a few employees had exhibited an onset of illness before the patrons, suggesting a possible source of infection; (8) the outbreak was biologically plausible – even if highly unlikely; and (9) failure to locate a source is not unusual, even in highly investigated outbreaks. Although one of the initial reasons to exclude terrorism (i.e., no prior incidents) is no longer applicable, determining if an unusual outbreak is the result of a deliberate act will remain a challenge. In this context, it is important to remember that the index case of inhalation anthrax identified in Florida in 2001 was initially thought to be a natural occurrence.

There are a number of epidemiologic clues that have been proposed that alone or in combination may suggest that an outbreak is deliberate. These indicators (**Table 4**) are based on distinctive epidemiology and laboratory criteria of varying specificity to evaluate whether an outbreak may be of deliberate origin. The clues focus on aberrations in the typical characterization of an outbreak by person, place, and time, in addition to consideration of the microorganism. Some of the clues, such as a community-acquired case of smallpox, are quite specific for bioterrorism whereas others, such as genetically similar microorganisms, may simply denote a natural outbreak. Evaluation (and confirmation) of a combination of clues increases the probability of determining events that result from an intentional release.

Epidemiologic clues can only be assessed in the context of a rapid and thorough epidemiologic investigation. Surveillance to identify increases in disease incidence is the first step and the cornerstone of bioterrorism epidemiology. However, even the most specific of epidemiologic clues (**Table 4**) may signal a new natural outbreak. For example, a community outbreak of individuals with smallpox-like lesions in the Midwest in 2003 may have indicated the deliberate release of smallpox virus. However, a thorough integrated epidemiologic and laboratory investigation identified the disease as monkey pox, an exotic disease in the United States, which in and of itself may suggest bioterrorism. In this outbreak, affected individuals were infected via prairie dogs purchased as pets, which had acquired their infection while cohoused with infected Giant Gambian rats that had recently been imported from Ghana. Therefore, the evidence did not support deliberate dissemination. Similarly, other emerging infectious diseases such as West Nile encephalitis and SARS would appropriately meet the minimal criteria for suspect bioterrorism and require a thorough investigation. Distinguishing natural outbreaks from those that are the result of an intentional attack requires an in-depth understanding of endemic diseases and their

Table 4 Epidemiological indicators of an intentional bioterrorism attack

- A single case of disease caused by an uncommon agent (e.g., smallpox, viral hemorrhagic fever, inhalation or cutaneous anthrax, glanders) without adequate epidemiologic explanation
- The presence of an unusual, atypical, genetically engineered, or antiquated strain of an agent (or antibiotic resistance pattern)
- Higher morbidity and mortality in association with a common disease or syndrome, or failure of such patients to respond to usual therapy
- Unusual disease presentation (e.g., inhalation anthrax, pneumonic plague)
- Disease with an unusual geographic or seasonal distribution (e.g., plague in a nonendemic area, influenza occurring in the Northern Hemisphere in the summer)
- Stable endemic disease with an unexpected increase in incidence (e.g., tularemia, plague)
- Atypical disease transmission through aerosols, food, or water, in a mode suggesting sabotage (i.e., no other possible explanation)
- Commonalities in diseased individuals (e.g., illness seen in persons who are in proximity and are exposed to a common ventilation system)
- Combination of diseases in a person or a cluster lacking precedent, or otherwise unexpected
- Unusual illness that affects a large, disparate population (e.g., respiratory disease in a large heterogeneous population may suggest exposure to an inhaled biological agent)
- Illness that is unusual (or atypical) for a given population or age group (e.g., outbreak of measles-like rash in adults)
- Unusual pattern of death or illness among animals (which may be unexplained or attributed to an agent of bioterrorism) that precedes or accompanies illness or death in humans
- Unusual pattern of death or disease in humans that precedes or accompanies illness or death in animals (which may be unexplained or attributed to an agent of bioterrorism)
- Ill persons who seek treatment at about the same time (point source with compressed epidemic curve)
- Agents isolated from temporally or spatially distinct sources have a similar genotype
- Simultaneous clusters of similar illness in noncontiguous areas, domestic or foreign
- Large numbers of unexplained diseases or deaths

epidemiology, and presents a significant challenge for surveillance and detection systems.

The most likely responders to an overt release of a microorganism or toxin (including hoaxes) are law enforcement or hazmat personnel and firefighters because of their terrorism training. Evaluations are made to assess the threat to public health, safety, and security. Once the threat assessment is complete, biological evidence is collected, preserved, and sent to a laboratory for analysis. Within the laboratory, a cadre of analytical tools is available to characterize the evidentiary material. Microbial forensics is focused on tracking and linking microorganisms to individuals and locations. Thus, different strategies may be used depending on the type(s) of evidence collected. In an overt attack, for example, the package, the biological agent and associated materials, as well as traditional forensic evidence (e.g., hairs, fibers, and fingerprints) may be analyzed. Therefore, an analytical plan may involve many different and diverse strategies. In a covert attack, evidence may be limited to patient diagnoses, epidemiologic data, and isolates from the victims.

Microbial Forensics Tools

Microbial forensics laboratories may perform detailed characterization assays to identify clues to the origin of a pathogen or toxin and/or its preparation for use in a criminal act. All pertinent evidence can be exploited. In addition to microbiological analyses (e.g., culture), the evidence collected from an overt event is likely to be amenable to chemical and physical analyses that may yield information about the methods, means, processes, and locations involved in the preparation and dissemination of the microorganism or toxin. The type of evidence found at the scene of an overt event might include powders, liquids, food, or other environmental samples. In addition, traditional forensic evidence (such as hair, fibers, documents, fingerprints, human DNA, etc.) also may be collected and analyzed.

A number of questions that could potentially be addressed through the genetic analysis of forensic samples are listed in **Table 5**. Defining the questions provides direction for an investigation, and the answers may provide information that can be used for attribution purposes or to provide investigative leads. The degree to which these questions can be addressed depends on the context of the case and the available knowledge of the genetics, genomics,

Table 5 Microbial forensics questions addressed by genetic analysis

- What might be deduced concerning the nature and source of the evidentiary sample?
- Is the pathogen detected of endemic origin or introduced?
- Do the genetic markers provide a significant amount of probative information?
- Does the choice of markers allow the effective comparison of samples from known and questioned sources?
- If such a comparison can be made, how definitively and confidently can a conclusion be reached?
- Are the genetic differences too few to conclude that the samples are not from different sources (or lineages)?
- Are these differences sufficiently robust to consider that the samples are from different sources?
- Is it possible that the two samples have a recent common ancestor or how long ago was there a common ancestor?
- Can any samples be excluded as contaminants or recent sources of the isolate?
- Are there alternative explanations for the results that were obtained?

phylogeny, and ecology of the microorganism in question. Genetic markers that may be useful for forensic attribution include single nucleotide polymorphisms (SNPs), repetitive sequences, insertions/deletions (INDELS), mobile genetic elements, pathogenicity islands, virulence and antimicrobial resistance genes, housekeeping genes, structural genes, and whole genomes. Many of these markers have been used for the molecular subtyping (i.e., genotyping) of pathogens where public health and epidemiological needs are foremost. Thus, developments in molecular epidemiology will contribute to the analytical toolbox of the microbial forensic scientist. The essential components of a highly precise and robust subtyping system for an infectious agent often are the same for both forensic and public health/epidemiologic purposes. These include (1) identification of diversity; (2) development of validated and robust molecular typing assays; (3) development of reference population databases; (4) establishment of guidelines for interpretation of analytical results, either qualitative or based on theoretical and probabilistic approaches; and (5) validation of the system with studies of actual disease outbreaks.

Analysis of Biological Signatures

Nucleic acid-based assays

Nucleic acid-based assays for microbial identification, characterization, and attribution purposes are widely used in the forensic arena to associate (and exclude) DNA-containing biological evidence with suspected sources. Ideally, for attribution, the forensic scientist attempts to narrow the possible sources of a sample while excluding most if not all other sources. This level of individualization may not always be achievable. To enhance attribution capabilities, a major thrust of microbial forensics is the use of nucleic acid-based assays that enable association (or elimination) of a pathogen with specific sources on the basis of genetic information from the full or partial genome of that pathogen. The use of these assays is analogous to the role they play in human DNA forensic analysis, where they are used on biological evidence to associate or exclude suspected individuals. However, the nucleic acid-based methods currently used in microbial forensics cannot routinely achieve the level of attribution that is achieved with human DNA forensics. The vast numbers of microorganisms, their complex biological and ecological diversities, and their capacity for genetic exchange complicate the analysis and interpretation of evidence in ways that do not impact human DNA forensics.

Prior to the introduction of the polymerase chain reaction (PCR), nucleic acid-based microbial identification was limited to techniques such as hybridization and typing by restriction fragment length polymorphism (RFLP) analysis. Such techniques required relatively large amounts of nucleic acid and were laborious, time-consuming, and not amenable to automation. The advent of PCR led to the development of sequence-specific amplification methods and a broad range of techniques that may ultimately meet the needs of the microbial forensics community.

PCR is typically thought of as a front-end assay component, whereby the PCR end products are assayed to determine the genetic profile of the sample. This notion changed with the advent of real-time PCR, which has become the genetic typing method of choice for characterizing microbes. The primary benefits of real-time PCR assays are the extremely low limits of detection (approaching single copy detection) and inherent specificity that enables the design of highly discriminating assays that are capable of distinguishing closely related molecular species. Further advantages of using this technique include (1) a broad dynamic range of quantification (up to seven orders of magnitude); (2) a short turnaround time (as little as 30 min); and (3) a reduction in laboratory contamination (assay is contained within a closed tube). The capacity to achieve unambiguous pathogen identification in as short a time as possible is broadly recognized as a key component in microbial forensic applications, public health investigations, and biodefense.

Various methods are used to identify markers that are targeted in real-time PCR assays. The most effective approach for comprehensive genetic variation discovery is high-throughput shotgun sequencing used by large genome centers. Whole genome sequencing is the preferred method for discovering genetic variation of value to forensic analysis. The power of this approach was demonstrated by a comparison of the genome sequence of *B. anthracis* Ames isolated from one of the victims of the 2001 bioterrorist anthrax attack to the genome sequence of a reference *B. anthracis* Ames strain. This comparison led to the identification of 60 polymorphic loci within the *B. anthracis* Ames genome that were comprised of SNPs, INDELS, and tandem repeat motifs. A number of these markers were found to separate a collection of anthrax isolates into distinct families. Although several genomes were sequenced to discover variants of the Ames strain of *B. anthracis* in the ongoing FBI investigation, it was a very costly process. Thus, current whole genome sequencing methodology is unlikely to be used routinely in biocrime cases. The relatively high costs of genome sequencing will limit its use for screening large repositories and population studies, until some of the newer high-throughput sequencing technology capabilities are realized.

If a reference sequence is available, resequencing using microarrays is an inexpensive, high-throughput alternative to whole genome sequencing. With proper microarray design, a large number of parallel analyses can be carried out simultaneously, and thousands of assays can be run at one time. Thus, a substantial increase in throughput can be realized, and genomes of many samples can be scanned rapidly. High-density array chips are being used to discover microbial polymorphisms, define population diversity, map virulence and antimicrobial resistance genes, and identify pathogens of consequence. The problem has been that mobile genetic elements and genes (including plasmids) can move horizontally between species and possibly confound species identification. However, because of the high-density oligonucleotide array on a chip surface, multiple 'unique' regions of any species, strain, or isolate can be typed so that confidence in a correct identification is increased over results from assays that type only one or a few regions of a genome.

The chip approach provides the capability to screen simultaneously a large number of microorganisms and affords a sensitivity level desirable for forensic demands. Resequencing arrays were designed to analyze over three million bases of the *B. anthracis* genome based on a panel of 56 strains. Replication studies showed very high sequence quality that was comparable to that obtained by shotgun sequencing. Chip technology will enable resequencing in a short period of time while screening multiple pathogens with high sensitivity, specificity, and nearly complete genome coverage. Not only will species and strain detection be possible, but chips also offer the possibility of detecting genetically engineered or modified strains through the detection of genes encoding antibiotic resistance or toxins as well as other heterologous genes. This application is obviously valuable for forensic attribution, but also has tremendous public health and homeland security applications.

The genotyping of biothreat agents presents a challenge when compared with other infectious disease-causing microorganisms in that the genetic relationship between many medically important microorganisms has been determined based on SNPs, the presence of known virulence factors, and macro restriction patterns of the genomic DNA. However, agents such as *B. anthracis*, *F. tularensis*, *Yersinia pestis*, and *Brucella* spp. are relatively monomorphic and exhibit little molecular variation among isolates from similar geographic areas. Thus, the identification and use of rare variants will be significant for obtaining the deepest resolution possible. Ongoing efforts to sequence multiple strains of these biothreat agents will facilitate the development of nucleic acid-based assays for attribution purposes.

A nested hierarchal strategy for subtyping these agents has been proposed. Progressive hierarchical resolving assays type genetic markers based on stability. SNPs are generally very stable and good for lineage studies, but tend to have a lower power of resolution for isolate individualization. Variable number tandem repeat (VNTR) loci (including microsatellites) are less stable and tend to evolve more rapidly; therefore, these loci (similar to human DNA forensic typing) will be more useful for distinguishing between similarly related samples.

A popular sequence-based bioinformatics approach that is used in attempts to establish the history and 'uniqueness' of a bacterial strain is the analysis of phylogenetic relationships. Bacterial phylogenies can be elucidated via the comparison of conserved regions of the genome. Phylogenetically informative gene sets include the ribosomal RNA genes that encode the 5S, 16S, and 23S rRNA genes and the intergenic spacer regions between these genes. The most widely used universal gene for phylogenetically positioning a sample (or for speciation) is the 16S rRNA gene. However, relying on a single region for speciation may not be sufficient and often is incapable of strain-level resolution. To gain further resolution, other conserved or universal genomic regions can be examined.

Multilocus sequence typing (MLST) analyzes 450–500 bp internal fragments of a minimum of five to seven housekeeping genes common to many bacteria. Most, if not all, bacterial species studied to date can be uniquely characterized using MLST. Data profiles of isolates are compared with those housed in a database. With proper design, many strains within a given species are distinguishable. For example, MLST has been useful in differentiating *B. anthracis* from its near-neighbors and in validating assays capable of differentiating virulent, vaccine, and avirulent *B. anthracis* strains. The real-time PCR assays used in these analyses are also capable of detecting *B. anthracis* pXO1 or pXO2 virulence plasmid markers that may reside in near-neighbor *Bacillus cereus* clade members. The advantage of MLST is that sequence data are readily exchangeable among laboratories. Thus, standards and databases, much like those for human forensic DNA analyses, can be generated.

Interpretations of genetic data from a clinical isolate, a laboratory-derived strain, or a reference database and its similarity to an evidence sample can be made quantitatively or qualitatively. Quantitative assessments convey the significance of an analytical result and rely on extant diversity data. Because of the lack of diversity data and limited knowledge of worldwide diversity in many cases, there can be a high degree of uncertainty associated with findings of a 'matched' (or similar) sample with some reference sample. While most scientists are becoming accustomed to statistical assessments of their data, this uncertainty will limit the use of some quantitative interpretations of microbial forensics results. However, qualitative assessments are also useful. They can provide direction for an investigation or indicate those samples that are dissimilar and could not be recently related to the evidence in question.

Non-nucleic acid-based analyses

DNA (or RNA) typing alone may not enable identification to the level of individualization, or at least to a very limited number of potential sources. Therefore, chemical and physical analyses of microbial forensic evidence may increase the likelihood for attribution. These types of signatures can only be obtained from crimes where the weaponized material or delivery device is found; they have little use in covert attacks where only the biological agent is derived from victims. The chemical characterization of a microorganism or its matrix may assist an investigation by providing information regarding the processes used to grow the agent and when or where it was grown. The sourcing application of stable isotope ratios has been used to address forensic questions such as determining the point-of-origin of illicit drugs. Like other organisms, microorganisms carry records of aspects of their growth environment, which consists primarily of nutrients and water, in the stable isotope ratios of their organic compounds.

An investigation of isotopic ratio analysis was performed using *Bacillus subtilis* grown in media prepared with water of varying oxygen (^{18}O) and hydrogen (^{2}H) stable isotopic ratios. The isotope ratios of the organic matter in spores harvested from these cultures were measured using a mass spectrometer and shown to be linearly related to those of the water used to prepare the growth medium. In addition, the predicted stable isotope ratios of spores produced in nutritionally identical media prepared with local water from five different locations (with different stable isotope ratios) around the United States matched the measured values of the water sources within a 95% confidence interval. Thus, stable isotope ratio analyses may be a powerful tool for tracing the geographic point-of-origin for microbial products, including vegetative cells and spores. However, only 30% of the hydrogen atoms in spores originated from the water used in the culture medium; the remaining 70% originated from the organic components of the culture medium, which may make the interpretation of the data for geo-location far more complex.

The stable isotope ratios (^{13}C, ^{15}N, ^{2}H) of microbiological culture medium vary based on the biological sources of the medium components – the most important being the C_3 and C_4 plants that are either a direct source of medium components or the base of the food chain for animal or yeast sources. Based on studies with *B. subtilis*, it has been suggested that the growth of microorganisms in different media should produce differences in microbial isotope ratios, which are readily measurable. Furthermore, the analysis of stable isotope ratios of microbiological agents and seized culture media would make it possible to rule out specific batches of media as having been used to culture a specific batch of microorganisms. These investigators also developed a model, using *B. subtilis*, that relates the hydrogen isotope ratios in culture media, water, and spores that works well for spores produced during growth in nonglucose media. These concepts can be extrapolated to other threat agents; however, differences in physiology and metabolism will require that these methods be validated with each microorganism.

A number of other technologies can be applied to microbial forensics. A number of these technologies have been used to examine weaponized spore preparations of *Bacillus globigii*, a commonly used surrogate for *B. anthracis*. Scanning electron microscopy (SEM) coupled with energy dispersive X-ray microanalysis (EDX) was used to determine the elemental composition of single cells or spores. SEM–EDX was able to detect the presence of silicon, of interest due to its use as a potential additive in the preparation of weapons grade *B. anthracis* endospores. Atomic force microscopy (AFM), which provides a high resolution image of the cellular surface, can be used to provide information on molecules adhering to the spores as well as on modifications to the exosporium caused by mechanical or chemical treatment during the weaponization process. Through the use of particle (proton)-induced X-ray emission (PIXE), and scanning transmission ion microscopy (STIM), the distribution of elements can be mapped within regions of a single cell. Raman and surface-enhanced Raman spectroscopy (SERS) probes molecular bond vibrations and rotations to produce characteristic spectra. This methodology has been used to distinguish between species of spores by probing as little as the first few nanometers of the spore surface. Bio-aerosol mass spectrometry (BAMS) has been developed for the rapid identification of individual cells or spores in an aerosol containing many background materials in real-time and without reagents. The mass spectrometry signature reflects both the intrinsic biologic agent and the matrix material in which it is embedded or coated. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) captures elemental data (to generate chemical maps) as well as molecular fragments (to generate mass spectra) in a depth-dependent basis (to generate a depth profile). This complementary technology can also detect the signatures of silica and other additives. Accelerator mass spectrometry (AMS) combines mass spectrometry and nuclear detection to measure the concentration of an isotope in a sample. AMS reduces the entire sample (less than 1 mg) to carbon before performing the analysis and provides the ^{14}C measurements on the bulk sample. This can be used to determine the age of the material (i.e., when the biological agent was prepared). Finally, mass spectroscopic methods (e.g., liquid chromatography/mass spectrometry (LC/MS) and matrix assisted laser desorption/ionization time of flight (MALDI-TOF)) have also been applied to the detection and characterization of toxins.

Biocrime Investigations

National Microbial Forensics Network

The anthrax mailings illustrated how crucial it was to have a specialized response network in place that is able to quickly and efficiently respond to biocrimes and bioterrorism. A criminal investigation, including the collection of forensic information on the threat agent, cannot begin until an attack or threat of an attack has been identified. Indeed, the anthrax letter attack began as a covert attack and became overt with the identification of the anthrax-tainted letters. Regardless, whether an attack is overt or covert, officials representing public health or animal health will likely initiate the investigation. Therefore, evidence from a covert attack on humans (e.g., cultures) is usually first analyzed by a laboratory belonging to the Laboratory Response Network (LRN), which is composed of state and local public health laboratories and the CDC. Law enforcement personnel become engaged once a crime is suspected, and typically forensic scientists are brought in when it is confirmed that a crime has occurred.

Following the discovery of the anthrax letters, the United States government established a dedicated national microbial forensics system. As part of this system, the United States developed the National Bioforensics Analysis Center (NBFAC), which is part of the National Biodefense Analysis and Countermeasures Center (NBACC) and the National Interagency Biodefense Campus (NIBC) located at Fort Detrick. The NBFAC, in conjunction with the partner laboratory network, serves as the national forensic reference center for the attribution of biological weapons in support of homeland security. Primary support for the NBFAC is provided by the DHS in partnership with the FBI.

In response to the threat of terrorism, part of the FBI's response and preparedness strategy was the establishment of the Chemical and Biological Sciences Unit (CBSU) within the FBI Laboratory in 2002. The CBSU is tasked with the responsibility of managing the scientific analyses of cases involving suspected threat agents (biological, chemical, and radiological/nuclear). The CBSU is subdivided into the Biology, Chemistry, and Infrastructure programs. The Biology and Chemistry programs perform research and development on methods and protocols not only in support of ongoing casework, but also with the goal of addressing attribution in the event of future attacks with chemical or biological weapons. The Infrastructure program acquires the facilities and equipment needed for the analysis of hazardous materials at partner laboratories and manages the training of a selected number of FBI laboratory examiners, already qualified in the more traditional aspects of forensics (trace, fingerprints, chemistry, DNA, etc.), for the Hazardous Evidence Analysis Team (HEAT). If the CBSU determines that traditional forensic analysis is needed on evidence collected from a crime scene, the evidence is directed to a partner laboratory that is equipped to handle that particular threat agent, and HEAT members are deployed to perform their examinations. The evidence is then sent to the NBFAC or other partner laboratories for pathogen identification and analyses using their in-house protocols or methods developed by CBSU.

Gaps in Microbial Forensic Analyses

The large variety of possible attack scenarios mandates the need to configure and prioritize a broad range of research and development efforts. With the ultimate goal of source attribution, a number of key areas must be addressed – (1) evidence collection, handling, and storage; (2) extraction and purification of trace nucleic acid, protein, or other signatures from samples; (3) methods for the identification of unique biological signatures (e.g., molecular genetics, microbiology, and cell biology); and (4) methods for the evaluation of the significance of biological data (e.g., genomics, informatics, and statistics).

Evidence collection, handling, and storage

The collection and preservation of microbial forensic evidence are paramount to successful investigation and attribution. If evidence (when available) is not collected, degrades, or is contaminated during collection, handling, transport, or storage, the downstream characterization and attribution analyses may be compromised. Retrieving sufficient quantities and maintaining the integrity of the evidence increase the chances of successful characterization of the material to obtain the highest level of attribution possible. There are a large variety of pathogens that could be used as bioweapons, not to mention the greater variety of environmental organisms that, if present as 'contaminants', might be of value in reconstructing the history of the event. This diversity precludes the creation of a single standard collection and preservation procedure that would be a catch-all for any and all possible organisms or toxins. However, a standard procedure(s) that encompasses the bacterial types that are most likely to be encountered can be created, with the understanding that additional information or an unusual locale might entail alteration of the procedure on a case-by-case basis.

Extraction and purification of biological signatures

Perhaps the most important aspect of the investigation occurs once samples reach the laboratory after collection – extraction of biological signatures. The focus here is on nucleic acid extraction since current protocols are heavily biased in favor of nucleic acid signatures, but the observations apply equally to other signatures such as proteins or fatty acids. The lack of proper cell disruption and nucleic acid extraction negates the use of successful collection and preservation and precludes the need for analytical and evaluation tools. As such, extraction techniques are a cornerstone of this new discipline. In this instance, variety is not the complicating factor. While there are a large variety of organisms, many of them fit into a few well-defined categories. Disruption and extraction techniques can be developed and validated across a spectrum. Many different extraction and 'nominal extraction' techniques are currently in use. A difficult task will be setting acceptable standards that the microbial forensics community can agree upon and will agree to adopt for extraction methodologies. However, development of extraction techniques is not the only factor that must be considered. All forensic disciplines must face the challenge of limited sample size. The same is true for microbial forensics. A common misconception is that a large amount of material is readily available through expansion of the sample via growth *in vitro*. In actuality, it is possible that the evidence is nonviable or unculturable in the laboratory. These possibilities must be considered when developing both disruption/extraction and identification protocols.

Identification of 'unique' signatures

Comparative genomic sequence characterizations are needed to identify the degree of variation, rates of mutation, and the extent of sequence divergence within known and questioned isolates or microbial groups. Also, there is a need to identify virulence and antibiotic resistance genes that could be targets for genetic manipulation or for selection of spontaneous antibiotic resistance. Because bioengineering capabilities are readily accessible, genetic engineering could be appealing to state sponsored programs and some individual bioterrorists. Using recombinant DNA technology, microbes can be readily modified, such that they can become more infectious or pathogenic, expand their host range, avoid host immune responses, and/or be made resistant to current medical countermeasures. Identifying signatures of purposeful manipulation, such as incorporation of an antibiotic-resistant gene, will become of utmost importance in determining whether an engineered microorganism was used as a bioweapon (or differentiating naturally occurring outbreaks of infectious diseases from intentional acts).

Data interpretation

Interpretation of results in a forensic analysis often entails a comparison of an evidence sample and a reference sample(s). There are three general categories of interpretation: inclusion (or association), exclusion, and inconclusive. An inclusion, or association, is stated when the pattern or profile from the two compared samples is sufficiently similar so that they potentially could have originated from the same source (or have a recent common ancestor). An alternate definition of an inclusion is a failure to exclude the two samples as having a common origin or belonging to the same group. An exclusion is stated when the pattern or profile is sufficiently dissimilar such that the two samples could not have originated from the same source. An inconclusive interpretation is rendered when there are insufficient data to provide a conclusive interpretation.

When the interpretation favors inclusion, or association, or the samples belong to the same genetic lineage, it is desirable to attach significance or weight to these results. However, care must be taken not to interpret the evidence beyond the limits of the assay and available data. Statistical inferences based on the strength of the inclusion or association depends on the relative information content of the genetic site(s) detected by the method(s) employed and extant supporting data.

Currently, there are few established statistical interpretation guidelines for microbial forensics data. In contrast, statistical assessment of human DNA forensics has been a well-studied area of investigation. Thus, one might follow the established protocols of human DNA forensic evidence interpretations for microbial forensics situations. Although the three general classes of observations (i.e., inclusion, exclusion, or inconclusive) are similar for human DNA and microbial forensics, there are some inherent differences, which must be invoked for statistical interpretation of the microbial forensic evidence.

For interpretation of analytic results, a better, more unifying statistical framework must be developed and tested for the comparison of forensic (and epidemiological) samples. This framework should be based on using lineage-based models where the extent of match/nonmatch evidence is assessed under the hypotheses of a particular sample(s) belonging to one group (or lineage) of putative samples or not. In other words, sequence similarity and/or a genotypic match may only infer a common lineage instead of unique identity. This possibility necessitates a somewhat different type of reference database for microorganisms, compared with those used in human DNA forensics. For example, in the human context, populations of diverse anthropological affinities have been collated and examined to investigate intra- and interpopulation variation of allelic and genotypic frequencies for autosomal loci and to study the extent of haplotype diversity (for mtDNA-sequences and Y-chromosome linked marker panels). Such data make it possible to derive a conservative estimate of the chance of a coincidental match (conditional or unconditional). In contrast, a genotypic match (albeit character state identity) or sequence similarity between microbial specimens, which are indicators of their evolutionary closeness, cannot be readily translated into a frequency; the reference data may not reflect a single population, nor provide any indication as to how many populations they could represent. Molecular evolutionary tools that can translate sequence (or genotype) similarity into evolutionary distance (or proximity) exist, which in turn can provide a signature of the most recent common ancestor of contrasted samples.

However, such molecular evolutionary tools may have to be refined for microbial forensics. For example, horizontal transfer of ecologically relevant genes, gene conversion, and recombination could uncouple the relationship between phylotypes (phylogenetically distinct). Thus, effective horizontal gene transfer, vertical gene transfer, and recombination statistical models, as well as general and specific genome site stability studies, near neighbor analyses, and diversity studies, will enhance capabilities to quantitatively assess the significance of lineage-based comparisons. Computational power and confidence will depend on the number of markers and their diversity within and among species, and the number of representative samples. Refined analyses will likely be based on coalescence theory incorporating population size fluctuation over generations, intersite variation of mutation rate, recombination, horizontal gene transfer (or gene conversion), and conservation of sites (based on function). Although it may be impossible to predict the complete diversity of microorganisms and their strains in any one geographic area, one could use collections of geographically diverse isolates from disease outbreaks. Isolates that cause disease may be relevant for population diversity estimates based on infectivity, host polymorphisms, and biogeography. Also, the assumption of a molecular clock (i.e., constancy of the rate of substitution over time), which is intrinsic in most algorithms of molecular evolution, has to be relaxed, since depending on the mode and medium of culture, temporal rate differences may be created either naturally or through engineering.

There is one notable difference between the statistical assessment of microbial forensic evidence and that used with human DNA forensics. In the human context, the courts and juries are accustomed to hearing about frequencies of a coincidental match such as one in a one hundred billion or less (or likelihood ratios on the order of billions or higher). Even with lineage-based markers, such as mitochondrial DNA or those residing on the Y chromosome, the estimates are in the orders of hundreds to thousands. In microbial forensics, time estimates of a more recent common ancestor will often be associated with standard errors that may be as large as the estimate (generally the coefficient of variation of the estimate, ratio of standard deviation, and the estimate would rarely be below 30%), or the bootstrap confidence of any lineage clustering may not exceed the complement of the inverse of the number of replications of the algorithm in the toolbox. As a consequence, the inherent relatively modest statistical support of any microbial forensic evidence should not be regarded as a drawback. But it is important that whatever statistic is calculated is done so within the limits of the genetic marker(s) and extant data. The statistical toolbox might also contain a quantitative guideline of what constitutes a ‘high’ versus ‘modest’ level of confidence in terms of the forensic implication of the evidence, rather than simple use of the nominal levels of significance used in applied statistics.

Conclusion

The challenges to developing a robust microbial forensics program are immense, but not impossible. The foundations are already in place and owe their origins to the field of epidemiology. Technology is developing to better recover evidence and purify it for downstream analyses. In the not too distant future, whole genome sequencing may become facile and relatively inexpensive; then, drilling down for rare SNPs in a genome for attribution purposes may become routine for even large-scale analyses. Bioinformatics tools and databases are developing and population genetic studies are underway so that the interpretation or significance of results can evolve from solely qualitative to quantitative assessments. Because it may not be possible to individualize microbial forensic evidence to the level enjoyed in human DNA forensics, non-nucleic acid-based analyses will figure prominently in scientific investigations, at least for overt attacks. For the foreseeable future, the field of microbial forensics will continue to mature in its capabilities so that law enforcement can provide the best services possible to deter, interdict, respond to, and investigate acts of bioterrorism.

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Gastrointestinal Microbiology in the Normal Host

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Defining Statement
Problems Associated with the Study of Gastrointestinal Flora
Techniques for Study of Gastrointestinal Flora
Normal Flora of Esophagus
Normal Flora of Stomach
Normal Flora of Small Intestine
Normal Flora of Colon
Addendum
Further Reading

Glossary

DNA	Deoxyribonucleic acid.
GI	Gastrointestinal.
OTU	Operational taxonomic unit, or molecular species.
PCR	Polymerase chain reaction.
phylotype	Group of organisms sharing a specific genetic constitution; species.
RNA	Ribonucleic acid.
16S rRNA	16 subunit of ribosomal ribonucleic acid.
UCLA	University of California, Los Angeles.

Abbreviations

DGGE	Denaturing gradient gel electrophoresis
FISH	fluorescent *in situ* hybridization
GI	gastrointestinal
OTU	operational taxonomic unit
PFGE	pulsed-field gel electrophoresis
TGGE	temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism

Defining Statement

The microflora of the human gastrointestinal tract has a profound influence on health and disease. Estimates indicate that the colon has 10^{14} bacterial cells, 10 times the number of tissue cells in the human body and containing at least 100 times as many genes as the human genome. Estimates of the number of species thought to be present in the human bowel vary from 1000 to 35 000 and 60–80% of these have not yet been cultivated.

Problems Associated with the Study of Gastrointestinal Flora

The composition of the gastrointestinal (GI) microflora is complex and many different methodologies have been used to study it. There is no optimum technique currently available. Many of these microorganisms that make up this flora are quite fastidious and are difficult (or impossible presently) to recover in culture, particularly if they are present in relatively small numbers. Nonetheless, even organisms in low counts may play an important role in physiologic and pathophysiologic processes. Ideally, specimens should be obtained in the laboratory (with a bathroom dedicated for this purpose); this is seldom possible. Thus, specimens usually need to be transported to a laboratory for study, sometimes over great distances. Since 99.9% of GI organisms are anaerobes, specimens should be transported to the laboratory under anaerobic conditions (which will usually not be a problem for aerobic organisms that are also present) and as expeditiously as possible. If the specimens cannot be delivered to the laboratory in less than 12–18 h, it is desirable to ship them under some form of refrigeration rather than by freezing the specimen. The distribution of different organisms in the fecal mass is irregular, so the entire bowel movement should be obtained and, upon arrival in the laboratory, homogenized under anaerobic conditions (or frozen until this can be done) before culturing or isolating and studying the DNA. Moisture content of stool specimens is also quite variable, so one should thoroughly dehydrate an aliquot with weighing before and after so that numbers of organisms of various types can be corrected to counts/dry weight.

Studies of GI flora are extremely time-consuming, but shortcuts in identification of isolates, or incomplete testing, may lead to gross errors. For cultural studies, a variety of media should be used, along with a variety of atmospheric conditions. Organisms vary greatly in their growth requirements. Unless selective and differential media are used, certain organisms present in lower counts may be overlooked. It is important to remember that many or most 'aerobes' are actually facultative and therefore can grow on anaerobically incubated plate media.

Older studies must be interpreted with caution as there may be a number of inaccuracies, alluded to above. There may also have been reclassification of organisms based on later knowledge and description of novel taxa previously unknown. It should also be recognized that normal bowel flora may be influenced by such factors as age, diet, geographic residence, and antimicrobial agents or other medications with some antibacterial activity. Sampling of certain areas of the small bowel may be very difficult or impossible. Tubes for such sampling will have less 'contamination' with local indigenous flora if they are passed via the nose, rather than through the mouth. Selective media, while very useful, always lead to suppression of certain organisms, sometimes including the ones sought with these media. One of the principal problems of culture-based techniques is the fact that the majority of the indigenous flora of the GI tract cannot be detected by such procedures. Most of these so-called 'uncultivable' organisms (a better term for them would be 'not yet cultured' organisms) are anaerobes or other very fastidious microorganisms. Molecular techniques provide the possibility to detect the majority of such organisms.

Molecular studies using microbial DNA may also be inaccurate if the stool or other specimen is not thoroughly homogenized before the DNA is extracted. In studying mixed populations, there may be insufficient or preferential cell lysis, inhibition of PCR, differential amplification or formation of chimeric or artefactual PCR products. In using 16S rDNA sequencing for identification, analysis of only 500 base pairs is not adequate for complete and accurate identification of many of the anaerobic organisms of the normal flora. The big problem with molecular approaches, of course, is that one does not recover the organism to do such important studies as pulsed-field gel electrophoresis (PFGE) for epidemiologic purposes, nor can one readily study antimicrobial susceptibility or virulence factors (although there are molecular techniques that are useful in this regard). In addition, recovery of DNA does not indicate whether the organisms from which it came were dead or alive (however, there are techniques that will give this information).

Quantitation of mucosal-associated populations is influenced by the washing procedure used to eliminate luminal organisms and reversibly-bound organisms. It is less of a problem with small bowel bacteria and others adhering to the epithelium than with colonic mucosa-associated flora where this flora is primarily in the mucus layer overlying the epithelium and may therefore be relatively easily lost during a washing procedure.

Techniques for Study of Gastrointestinal Flora

There are many protocols for cultural studies of GI flora. It is important that good anaerobic conditions are provided during transport of the specimens and for the culture and examination processes. With GI flora, many organisms are quite fastidious and may not survive if they are cultured in an anaerobic jar and then removed to an aerobic atmosphere while the plates are examined before subculturing. It is better to use an anaerobic glovebox or cabinet with an anaerobic incubator attached to it so that plates are never exposed to air. After an initial subculture or two, many organisms can be processed on a laboratory bench and then replaced into an anaerobic jar within 20 min or less. Others will require constant anaerobiosis. A good protocol makes use of prereduced nonselective enriched plate media (e.g., Brucella blood agar plates supplemented with vitamin K_1 and hemin) and numerous selective and differential media to facilitate recovery of particular types of organisms. The more carefully chosen selective/differential media utilized, the better. One must decide what is practical, but for study of indigenous flora the more the better. Serial dilutions of the specimens may be made in prereduced yeast extract solution in an anaerobic chamber. Accurate identification often will require sequencing of the 16S ribosomal and/or other genes in addition to phenotypic tests.

Various molecular approaches may be used and will provide a remarkable amount of additional information. Clone libraries, while tedious and time-consuming, have been used with excellent results. Terminal restriction fragment length polymorphism (T-RFLP) has been considered to be promising for analysis of communities such as GI flora but is somewhat lacking in precision. Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) and fluorescent *in situ* hybridization (FISH) have also been used effectively for community flora analyses. Probe grids or DNA checkerboard hybridization may be used and DNA microarrays are available, but not with a comprehensive array for GI flora. A preferred technique is real-time PCR. It requires that one have primer-probes for target sequences of groups of organisms or for each individual organism that may be present (which immediately places limitations since we do not yet know the total makeup of GI microflora communities). For studies of known organisms for which there are primer-probes there are high-throughput methods that provide quantitation as well as accurate identification, and detection of organisms that are present in small numbers. Pyrosequencing techniques are new and not yet highly developed but offer great promise for the future.

Normal Flora of Esophagus

It has generally been considered that the esophagus has no resident microflora, that bacteria occur there only transiently and represent swallowed organisms from the oral cavity. However, a paper in 1983, using older techniques, did find aerobic organisms in all subjects studied and anaerobes in 80% of subjects. The most common aerobes might have represented oral flora – *Haemophilus, Neisseria*, and streptococci and yeasts, but coliforms were found in half the subjects. The most common anaerobes encountered were anaerobic cocci and *Bacteroides*, including *Bacteroides fragilis*. In a very recent paper, careful studies were reported on seven subjects ranging in age from 40 to 75 years. One of these was a smoker, one had inflammation (severe) noted on endoscopy, and three were being treated with proton pump inhibitors; there may have been overlap with regard to these factors. None had been on antibiotics in the previous 4 weeks. Cultures were done on aspirated fluid and on mucosal biopsies (six from the lower third of the esophagus, one from the middle third). Both nonselective and several selective media were used for isolation and 16S rDNA sequencing (500 base pairs) was used for identification. Microbiotas were detected in aspirates from four subjects and in mucosal biopsy samples from three; three of the four subjects not receiving proton pump inhibitors were colonized by bacteria. The pH of aspirates ranged from 1.6 to 4.0 (mean 2.3). A total of 13 species of microorganisms were recovered, none from subjects whose pH was <2.0. Lactobacilli (8 species), streptococci (two species), and yeasts (two species of *Candida* and one of *Saccharomyces*) were the only organisms found in aspirate specimens. Mean counts of bacteria were $10^{2.3}$ to $10^{2.8}$ and of yeasts $10^{4.3}$. In mucosal tissue, only two lactobacillus species were obtained (mean count $10^{4.7}$), together with three species of streptococci (mean count $10^{4.2}$), and other organisms such as *Actinomyces, Bifidobacterium, Propionibacterium, Veillonella* and *Prevotella* (mean counts $10^{2.8}$ to $10^{3.5}$); no yeasts were recovered. Thus, bacteria in mucosal samples were more diverse and present in higher counts than in aspirates, but aspirates contained yeasts and mucosal samples did not.

Normal Flora of Stomach

In most fasting normal subjects, gastric juice is sterile, but there is significant colonization in the face of hypochlorhydria or achlorhydria of any cause. After meals, bacterial counts obtained from cultures may go as high as $10^6\ \mathrm{ml}^{-1}$ but the organisms are rapidly killed by gastric acid so that the stomach is sterile by 1 h. At pH values <2.0, gastric fluid is usually sterile, with a small percentage colonized by acid-resistant organisms such as yeast or lactobacilli.

However, an elegant study of gastric mucosal flora published recently gives quite a different picture. Single mucosal biopsies were taken from either the corpus of the stomach or the gastric antrum of 23 adults and subjected to analysis by using a small subunit 16S rDNA clone library approach. There were 1833 sequences generated by broad-range bacterial PCR, with 128 phylotypes identified. Five major phyla dominated – *Proteobacteria* (952 clones, 32 phylotypes), *Firmicutes* (low GC Gram-positive bacteria) with 464 clones and 36 phylotypes, *Bacteroidetes* (193 clones, 35 phylotypes), *Actinobacteria* (high GC Gram-positive bacteria) with 164 clones and 12 phylotypes, and *Fusobacteria* (56 clones, 10 phylotypes). The remaining sequences were in the TM7, *Deferribacteres*, and *Deinococcus*/*Thermus* phyla. *Helicobacter pylori* sequences constituted 42% of all sequences analyzed and 777 clones (in 7 of the 19 subjects positive for this organism, conventional tests for *H. pylori* were negative). The second most common genus found was *Streptococcus* (299 clones) and the third most common was *Prevotella* with 139 clones. Ten percent of the phylotypes had not been characterized previously. The microbial flora overall did not relate to the presence or absence of *H. pylori* or to the gastric location biopsied or the pH; it appeared to be distinct also from the floras of the subgingival crevice and esophagus when compared to previous studies.

Normal Flora of Small Intestine

Studies of the duodenum have either failed to yield any organisms or found only low counts (typically $10^2\ \mathrm{ml}^{-1}$), chiefly acid-tolerant organisms such as lactobacilli, streptococci, and yeasts. On occasion, other organisms such as *Bacteroides* spp., *Bifidobacterium* spp., *Veillonella* spp., *Staphylococcus* spp., and enterobacteria were found in very small numbers.

Jejunal flora is also sparse, but more specimens from the jejunum yield growth and in higher numbers than is true for the duodenum. Several studies employed aspiration of luminal fluid with needle and syringe at the time of surgery (without prophylactic antibiotics), so the data is quite reliable. Organisms recovered were similar to those found in duodenal flora, with lactobacilli (chiefly *Lactobacillus fermentum, Lactobacillus reuteri, Lactobacillus gasseri*, and *Lactobacillus salivarius*), streptococci, staphylococci, *Veillonella* spp., yeasts, and sometimes enterococci being detected. Homogenates of jejunal biopsies yielded a flora generally comparable to that found in luminal fluid. Specimens from subjects in

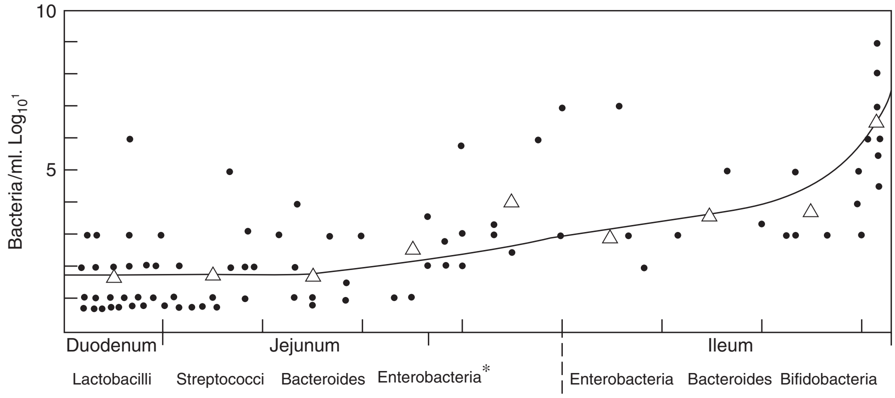

Figure 1 The distribution of viable bacteria in the small intestine. Reproduced with permission from Drasar S and Hill MJ (1974) *Human Intestinal Flora*, p. 40. London: Academic Press.

developing countries often had much higher counts and a more diverse flora.

In the ileum, peristalsis is slower than in the upper bowel and intestinal juice dilutes pancreatic enzymes and bile which have an antimicrobial effect. This results in a more profuse and diverse flora, related to both organisms coming down from above and reflux of cecal contents through the ileocecal sphincter. In the ileum, microbial counts are often in the range of 10^{6-8}, dominated by streptococci and enterococci, enterobacteria, lactobacilli, and other facultative bacteria but also including obligate anaerobes such as *Veillonella* spp., *Clostridium* spp., and *Bacteroides* spp., including *B. fragilis*. Other organisms, in smaller numbers, include *Bifidobacterium* spp., anaerobic cocci, *Fusobacterium* spp., *Eubacterium* spp. and staphylococci. A study of jejunal and ileal flora in three subjects, using terminal restriction fragment length polymorphism, obtained results similar to those cited above.

There is also a very recent study of terminal ileum (and colon, to be discussed in the subsequent section) from six subjects requiring emergency bowel resection (no antibiotic therapy used), using both denaturing gradient gel electrophoresis (DGGE) and real-time PCR. Organisms identified by PCR amplicon sequencing from the DGGE gels included *Bacteroides fragilis*, *B. vulgatus*, *Clostridium fallax*, *C. acetobutylicum*, *Vibrio campbelli*, *Desulfococcus multivorans*, *Mycoplasma faucium*, and two unidentified organisms. The real-time PCR studies revealed total counts of eubacteria significantly higher than in three colonic sites sampled. Counts of individual organisms or groups detected were as high as $10^{7.4}$ and mean counts of organisms ranged from $10^{2.2}$ to $10^{6.2}$. Organisms detected by real-time PCR included lactobacilli, enterobacteria, *Enterococcus faecalis*, *Bacteroides*, *Bifidobacterium*, *Desulfovibrio*, *Peptostreptococcus anaerobius*, *Clostridium clostridioforme*, *C. butyricum*, *Eubacterium rectale*, and *Faecalibacterium prausnitzii* (**Figure 1**).

Normal Flora of Colon

> "And it is truly wonderful that a substance, the very aspect and odor of which are sufficient to induce an inevitable nausea, should be regarded not merely as a matter of curiosity and study, but held in the highest repute as a unique and most precious treasure for the preservation of health."
>
> Samuel Augustus Flemming, 1738.

Succession of Flora in Infants

The GI tract is sterile at birth and becomes colonized with successive floras until a complex microbiota resembling that of adults is established by age 2. The newborn becomes colonized with microbes from the genital and anal tracts of the mother during delivery and then from breast milk and the mother's skin flora. During the first 24 h of life, bacteria found in the infant's feces include micrococci, streptococci, enterobacteria, lactobacilli, *Bacteroides* and clostridia. Between 2 days and 1 week, enterobacteria and streptococci dominate. In breast-fed infants, bifidobacteria predominate by 4 weeks and these persist until there is dietary supplementation. Formula-fed infants maintain a more complex flora. Both groups of infants have *Bacteroides* spp. (*B. vulgatus* and *B. fragilis*) and *Parabacteroides distasonis*. With the introduction of solid food, differences between formula-fed and breast-fed infants disappear. By 12 months of age, facultative anaerobes decrease and obligate anaerobes resemble those found in adults. Microbial flora in infants is much more variable than that of adults, in terms of counts, and relatively small changes in diet may produce major effects on the bowel flora.

Cultural Studies of the Microflora of the Adult Colon

One gram of feces contains significantly more bacteria than there are people on the earth. Usually the colonic flora is inferred from studies of feces. It is generally not practical, of course, to sample the bowel lumen directly. One such study was done, however, in ten patients undergoing elective cholecystectomy. Material obtained by needle aspiration of the cecum and transverse colon was studied only for total counts of various facultative bacteria and anaerobes and revealed counts of both that were somewhat lower than obtained from stool specimens from these subjects. In the most comprehensive and detailed studies of this type (**Tables 1–8**) a total of 141 adults had studies of fecal flora. Included were several different diet groups as well as some subjects with colonic polyps and early colon cancer. Differences in flora between these various groups were small with the procedures available at that time. *Bacteroides* were found in all subjects and in the highest counts of all organisms recovered; *B. thetaiotaomicron* was the species most commonly encountered but counts of the other *Bacteroides* recovered (*B. vulgatus, B. fragilis, B. ovatus* and (now *Parabacteroides*) *distasonis* were also high. The principal genera or groups recovered, in addition to *Bacteroides*, were anaerobic cocci, *Eubacterium*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and facultative streptococci and Gram-negative bacilli. Since this data was published, there have been many changes in nomenclature and taxonomy. Identification of organisms in this study was done only by phenotypic studies and it is now known that 16S rDNA (and other) gene sequencing gives more accurate identification (and is more rapid). A culture-based study of 20 Japanese-Hawaiian older adults, using the roll-tube technique, gave similar results. Studies of mucosa-associated flora are difficult to do but some organisms are associated with the mucosal epithelium and others with the overlying mucin layer. These bacteria are felt to be largely of the same genera as those found in the colonic lumen when cultural techniques are used. It is felt that the species diversity displayed in the colonic flora helps maintain a balance among the resident organisms.

There have been a number of newer phenotypic methods introduced since these studies were done that would provide greater accuracy of identification. In particular, study of preformed enzymes (commercial kits such as the

Table 1 Fecal microbiota in various dietary groups including Seventh-Day Adventists who were strictly vegetarian, Japanese who consumed an oriental diet that included fish but no beef, and healthy subjects who consumed a Western diet with relatively large quantities of beef

	Strictly vegetarian (13)		*Japanese (15)*[c]		*Western (62)*		*Total (141)*[d]	
Microorganisms	*%*[a]	*Mean*[b]	*%*	*Mean*	*%*	*Mean*	*%*	*Mean*
Bacteroides	100	11.7	93	10.8	100	11.3	99	11.3
Fusobacterium	0		40	8.1	24	8.6	18	8.4
Anaerobic streptococci	8	11.4	60	9.5	32	10.5	34	10.3
Peptococcus	8	11.2	47	9.4	37	10.1	33	10.0
Peptostreptococcus	23	11.1	80	10.2	35	10.2	45	10.1
Ruminococcus	54	10.2	60	10.3	45	10.0	45	10.2
Anaerobic cocci	85	10.3	100	10.7	98	10.6	94	10.7
Actinomyces	31	10.5	0		2	5.7	7.8	9.2
Arachnia-Propionibacterium	38	10.0	0		2	5.5	9.2	8.9
Bifidobacterium	69	10.9	80	9.7	79	10.4	74	10.2
Eubacterium	92	11.0	93	10.6	95	10.6	94	10.7
Lactobacillus	85	11.1	73	9.0	73	9.3	78	9.6
Clostridium	92	9.4	100	9.7	100	10.2	100	9.8
Streptococcus	100	8.6	100	8.7	100	9.1	99	8.9
Gram-negative facultatives	100	8.2	100	9.2	98	8.9	98	8.7
Candida albicans	15	4.9	47	5.6	14	5.4	14.2	5.4
Other yeasts	23	5.6	53	5.8	31	5.2	36.2	5.6
Filamentous fungi	0		0		3	3.8	3.5	5.9
Bacillus spp.	69	4.2	80	6.2	82	5.0	82.3	5.2
TOTAL[e]	100	12.6	100	11.8	100	12.2	100	12.2

[a] % Positive.

[b] Mean count expressed as organisms log 10/g dry-weight feces.

[c] Number of subjects per dietary group.

[d] Total for all 141 subjects including colonic polyp, colonic cancer, and vegetarians who consume some meat.

[e] Total of all microbes detected (including other genera and groups not listed above).

Data from Finegold, Sutter, and Mathisen 1983, modified by Conway PL Chap. 1 in Gibson GR and Macfarlane GT (1995) Human Colonic Bacteria. Boca Raton, FL: CRC Press; with permission.

Table 2 Gram-negative anaerobic rods recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Bacteroides										
B. amylophilus	0		0		0		2	9.0	1.4	7.6–9.0 (8.3)
B. capillosus	15	11.0–11.7 (11.4)	7	10.9	7	10.1	15	9.4–11.5 (10.4)	14.9	9.3–11.7 (10.5)
B. coagulans	8	10.0	0		0		3	10.8–11.3 (11.1)	3.5	8.7–11.7 (10.5)
B. distasonis	39	9.8–11.1 (10.5)	36	10.2–11.5 (11.0)	60	9.2–11.6 (10.3)	52	9.3–12.5 (10.6)	52.5	7.9–12.5 (10.5)
B. eggerthii	0		0		0		5	10.0–11.0 (10.5)	2.1	10.0–11.0 (10.5)
B. fragilis	23	8.9–12.0 (10.1)	50	9.9–11.7 (11.0)	33	8.4–10.9 (9.8)	50	7.5–11.8 (10.3)	46.1	7.5–12.0 (10.4)
Other species[d]	23	10.5–11.7 (11.0)	21	11.1–11.7 (11.4)	40	7.6–10.9 (9.8)	24	8.6–11.8 (10.4)	25.5	7.6–11.8 (10.5)
'Giant'[e]	15	8.5–10.2 (9.4)	14	8.6–10.7 (9.6)	0		6	8.8–11.4(10.2)	5.7	8.5–11.4 (9.9)
B. hypermegas	0		0		0		5	8.6–10.9 (9.8)	2.8	8.6–10.9 (9.8)
B. melanino genicus-asaccharolyticus group	0		0		7	9.6	0		3.5	8.3–10.7 (9.6)
B. oralis	0		0		0		2	10.2	1.4	10.2–10.4 (10.3)
B. ovatus	31	9.5–11.2 (10.3)	21	10.7–11.4 (11.1)	40	7.2–10.9 (9.6)	24	5.7–11.1 (9.7)	29.1	5.7–11.7 (10.0)
B. pneumosintes	0		0		7	9.7	7	10.2–10.6 (10.4)	8.5	7.5–11.7 (9.8)
B. putredinis	8	10.5	21	10.6–11.6 (11.1)	0		11	10.0–11.4 (10.6)	7.8	10.0–11.6 (10.7)
B. ruminicola group	15	10.7–11.4 (11.0)	7	11.6	13	8.8–10.7 (9.7)	18	9.8–11.4 (10.6)	15.6	8.8–11.6 (10.5)
B. splanchnicus	15	10.5–12.1 (11.3)	7	10.6	7	10.8	2	10.6	4.3	10.0–12.1 (10.8)
B. thetaiotaomicron	100	9.4–12.5 (11.3)	86	7.7–12.4 (11.4)	80	9.2–11.4 (10.2)	86	6.3–12.0 (10.3)	86.5	6.3–12.5 (10.7)
B. ureolyticus	0		0		0		2	10.9	0.7	10.9
B. vulgatus	69	9.5–12.2 (10.8)	57	10.0–12.0 (11.1)	47	8.8–11.4 (10.1)	71	8.6–13.5 (10.5)	69.5	8.6–13.5 (10.6)
Species[f]	31	10.5–11.7 (11.1)	36	9.5–11.7 (11.0)	40	7.6–10.9 (9.9)	50	1.3–11.8 (5.8)	47.5	1.3–11.8 (7.0)
Total count of *Bacteroides*	100	10.6–12.6 (11.7)	100	9.5–12.5 (11.8)	93	9.6–12.0 (10.8)	100	9.2–13.5 (11.3)	99.3	9.2–13.5 (11.3)
Fusobacterium										
F. gonidiaformans	0		0		20	5.9–6.7 (6.3)	3	6.6–6.7 (6.6)	3.5	5.9–6.7 (6.5)
F. mortiferum	0		0		0		5	9.6–9.9 (9.8)	2.8	7.2–9.9 (9.2)

(Continued)

Table 2 (Continued)

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
F. necrogenes	0		0		0		3	7.7–8.3 (8.0)	1.4	7.7–8.3 (8.0)
F. necrophorum	0		0		7	9.5	5	5.1–8.4 (7.3)	3.5	5.1–9.5 (7.4)
F. prausnitzii	0		0		0		2	8.4	1.4	8.4–8.6 (8.5)
F. russii	0		0		7	8.0	3	7.5–11.0 (9.3)	3.5	5.9–11.0 (8.4)
Species[g]	0		0		13	8.8–9.0 (8.9)	5	7.7–10.6 (9.5)	5	6.5–10.6 (9.0)
Total count of *Fusobacterium*	0		0		40	6.4–9.5 (8.1)	24	5.1–11.0(8.6)	18.4	5.1–11.0 (8.4)
Butyrivibrio										
B.fibrisolvens	0		0		0		0		0.7	7.5
Total count of Gram-negative anaerobic rods	100	10.6–12.6 (11.7)	100	9.5–12.5 (11.8)	100	6.7–12.0 (10.5)	100	9.2–13.5 (11.3)	100	6.7–13.5 (11.3)

[a] Total fecal flora studies were done on 141 subjects (Seventh-Day Adventists who were strict vegetarians, Seventh-Day Adventists who consumed only small quantities of meat, Japanese who consumed an Oriental diet that included fish but no beef, patients with colonic polyps, patients with nonobstructing colon cancer, and healthy subjects who consumed a Western diet that contained relatively large quantities of beef. The columns for patients with colonic polyps and colon cancer have been left out. Total is for all 141 subjects.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects studied.

[d] Other *B. fragilis* group species, aside from *B. distasonis*, *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, and *B. vulgatus*.

[e] Large forms not fitting recognized species.

[f] Fourteen isolates were recovered that could not be speciated using currently accepted identification protocols and presently recognized species. These appeared different enough to represent possibly 10 separate species.

[g] Ten isolates (possibly representing seven species) could not be speciated using currently accepted identification protocols and presently recognized species.

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 3 Anaerobic cocci recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
***Acidaminococcus** fermentans*	0		7	8.2	20	5.0–7.9 (6.3)	27	3.6–11.6 (8.7)	19.1	3.6–11.6 (8.5)
Acidaminococcus species[d]	0		7	8.2	20	5.0–7.9 (6.3)	23	3.6–10.7 (8.7)	18.0	3.6–11.1 (8.5)
Total count of *Acidaminococcus*	0		7	8.5	20	5.0–8.2 (6.6)	27	3.7–11.6 (9.0)	20.0	3.7–11.6 (8.8)
Anaerobic streptococci										
S. constellatus	0		7	10.6	7	11.3	2	4.7	4.3	4.7–11.3 (9.5)
S. hansenii	0		7	10.6	7	7.0	3	10.5–11.8 (11.1)	5.0	7.0–11.8 (10.5)
S. intermedius	8	11.4	7	12.0	47	8.8–10.4 (9.6)	31	6.4–12.6 (10.2)	27.7	6.4–12.6 (10.2)
S. morbillorum	0		0		0		3	9.4–10.4 (9.9)	1.4	9.4–10.4 (9.9)
Total count of anaerobic streptococci	8	11.4	14	10.9–12.0 (11.5)	60	7.0–11.3 (9.5)	32	8.6–12.6 (10.5)	34.0	7.0–12.6 (10.3)
***Coprococcus** comes*	0		14	3.8–10.9 (7.4)	0		8	9.3–12.0 (9.9)	5.7	3.8–12.0 (8.6)
Gaffkya	0		0		0		0		0.7	12.6
***Megasphaera** elsdenii*	0		0		13	8.3–10.0 (9.2)	5	8.7–10.5 (9.6)	5.0	8.3–10.5 (9.4)
***Peptococcus** asaccharolyticus*	0		7	10.7	20	8.0–9.9 (9.1)	3	10.4–12.0 (11.2)	5.0	7.5–12.0 (9.7)
P. magnus	0		0		0		7	5.1–11.0 (9.3)	6.4	5.1–11.7 (9.9)
P. prevotii	8	11.2	0		20	7.0–10.6 (9.3)	23	5.9–12.7 (9.9)	16.3	5.9–12.7 (10.0)
P. saccharolyticus	0		0		0		2	12.5	3.5	7.1–12.5 (9.3)
P. variabilis	0		0		0		3	9.4–10.8 (10.1)	2.8	9.4–10.8 (10.1)
Peptococcus species[e]	0		0		33	5.6–10.5 (8.3)	8	9.9–10.7 (10.3)	9.9	5.6–10.7 (9.4)
Total count of *Peptococcus*	8	11.2	7	10.7	47	7.0–10.6 (9.4)	37	5.1–12.9 (10.1)	33.3	5.1–12.9 (10.0)
***Peptostreptococcus** P. anaerobius*	0		0		0		2	8.6	1.4	8.6–12.3 (10.4)
P. micros	0		0		13	8.9–10.0 (9.4)	8	7.4–11.7 (9.9)	7.1	7.4–11.7 (9.5)
P. parvulus	0		0		0		8	5.6–11.3 (9.6)	4.3	5.6–11.3 (9.6)

(*Continued*)

Table 3 (Continued)

Organism	*Strictly vegetarian (13)*[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
	% positive	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
P. productus	15	10.2–11.3 (10.8)	50	5.3–11.8 (10.3)	40	7.0–10.9 (9.9)	18	5.4–12.6 (9.9)	29.8	3.8–12.6 (9.7)
Peptostreptococcus species[f]	8	11.7	0		67	8.8–11.7 (9.9)	8	10.0–11.0 (10.5)	15.6	8.5–11.7 (10.0)
Total count of *Peptostreptococcus*	23	10.2–11.7 (11.1)	50	5.3–11.8 (10.3)	80	9.2–11.7 (10.2)	35	5.4–12.6 (10.2)	45.4	3.8–12.6 (10.1)
***Ruminococcus** albus*	15	8.5–11.3 (9.9)	0		20	5.4–10.0 (8.3)	18	4.6–11.6 (9.0)	14.9	4.6–11.6 (9.2)
R. bromii	8	11.0	0		7	10.0	7	4.7–11.5 (9.1)	6.4	4.7–11.5 (9.8)
R. callidus	0		0		0		3	10.3–11.0 (10.7)	2.1	9.5–11.0 (10.3)
R. flavefaciens	0		0		0		3	9.8–10.6 (10.2)	2.1	9.8–11.9 (10.8)
R. lactaris	0		0		13	7.0–9.9 (8.5)	5	9.7–10.8 (10.3)	4.3	7.0–10.8 (9.7)
R. obeum	0		0		27	6.5–10.5 (9.3)	0		4.3	6.5–10.9 (9.5)
R. torques	0		0		7	10.5	0		1.4	8.9–10.5 (9.7)
Ruminococcus species[g]	46	7.8–11.4 (9.8)	36	10.3–11.6 (10.9)	20	9.9–11.0 (10.5)	24	6.4–12.7 (10.4)	25.5	6.4–12.8 (10.5)
Total count of *Ruminococcus*	54	7.8–11.4 (10.2)	36	10.3–11.6 (10.9)	60	9.4–11.0 (10.3)	45	4.6–12.7 (10.0)	45.0	4.6–12.8 (10.2)
***Sarcina** lutea*	0		0		7	3.9	0		0.7	3.9
S. ventriculi	8	7.5	7	7.5	0		0		2.1	7.0–7.5 (7.4)
Sarcina species[h]	8	7.5	7	7.5	7	3.4	2	5.6	5.7	3.4–8.6 (6.6)
Total count of *Sarcina*	8	7.8	7	7.8	13	3.4–3.9 (3.7)	2	5.6	6.4	3.4–8.6 (6.4)
Veillonella	62	4.9–11.6 (8.2)	29	10.6–12.6 (11.6)	33	6.7–10.0 (8.3)	34	3.5–13.4 (7.7)	34.0	3.5–13.4 (7.9)
Total count of anaerobic cocci	85	5.9–11.8 (10.3)	79	10.3–12.6 (11.6)	100	10.1–11.8 (10.7)	98	4.0–13.4 (10.6)	94.0	4.0–13.4 (10.7)

[a] For a description of subjects see footnote *a*, **Table 2**.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects studied.

[d] Twenty-six isolates.

[e] Nine isolates (eight species)

[f] Twenty-four isolates (13 species) could not be speciated using currently accepted identification protocols and presently recognized species.

[g] Four isolates (four species).

[h] Twelve isolates (three species).

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 4 Gram-positive non-spore-forming anaerobic rods recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Actinomyces										
A. naeslundii	23	9.2–10.9 (10.2)	29	5.8–11.0 (8.4)	0		2	5.7	5.7	5.7–11.0 (8.8)
A. odontolyticus	0		0		0		0		0.7	9.8
Species[d]	8	11.1	0		0		0		1.4	9.8–11.1 (10.4)
Total count of *Actinomyces*	31	9.2–11.1 (10.5)	29	5.8–11.0 (8.4)	0		2	5.7	7.8	5.7–11.1 (9.2)
Arachnia–Propionibacterium										
A. propionica	0		0		0		0		0.7	10.6
P. acnes	38	4.3–12.0 (10.0)	29	4.7–11.7 (8.4)	0		5	7.4–10.5 (9.1)	9.2	4.3–12.0 (9.4)
P. avidum	0		0		0		0		0.7	10.0
Species[e]	0		0		0		2	5.5	1.4	5.5–6.0 (5.7)
Total count of *Arachnia–Propionibacterium*	38	4.3–12.0 (10.0)	29	4.7–11.7 (8.4)	0		2	5.5	9.2	4.3–12.0 (8.9)
Bifidobacterium										
B. adolescentis group	62	9.5–12.2 (10.9)	43	7.6–11.5 (10.0)	53	8.0–10.4 (9.5)	52	5.7–13.4 (10.3)	54.6	5.7–13.4 (10.0)
B.bifidum	0		0		7	9.9	10	8.4–11.4 (10.3)	5.0	8.4–11.4 (10.2)
B. breve	0		0		33	7.6–9.9 (8.9)	15	7.6–10.8 (9.3)	9.9	7.6–10.8 (9.2)
B. eriksonii	0		0		27	8.2–10.6 (9.7)	5	9.4–10.7 (10.1)	5.7	8.2–10.7 (9.7)
B. infantis group	8	11.9	14	10.2–10.6 (10.4)	7	9.0	39	6.1–12.4 (9.9)	24.8	5.7–12.4 (9.8)
B. longum	15	9.8–10.6 (10.2)	7	11.6	13	8.6–9.3 (8.9)	24	9.1–11.3 (10.4)	21.3	4.9–11.6 (9.9)
Species[f]	0		7	11.9	7	11.3	2	9.4	2.1	9.4–11.9 (10.8)
Total count of *Bifidobacterium*	69	9.5–12.2 (10.9)	64	7.6–11.9 (10.4)	80	7.6–11.4 (9.7)	79	5.7–13.4 (10.4)	74.0	4.9–13.4 (10.2)
Eubacterium										
E. aerofaciens	46	8.0–11.8 (10.6)	57	3.5–11.5 (7.9)	47	6.3–11.1 (9.5)	42	5.9–12.5 (9.8)	48.9	3.5–12.5 (9.7)
E. alactolyticum	0		0		0		2	4.3	0.7	4.3

(*Continued*)

Table 4 (Continued)

Organism	*Strictly vegetarian (13)*[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
	% positive	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
E. biforme	15	8.3–11.3 (9.8)	7	9.9	7	9.3	8	8.6–9.9 (9.3)	7.1	8.3–11.3 (9.7)
E. budayi	0		7	5.4	0		0		0.7	5.4
E. cellulosolvens	0		7	10.4	0		0		0.7	10.4
E. combesii	0		0		0		8	7.5–11.4 (10.1)	4.3	7.5–11.4 (10.1)
E. contortum	54	4.8–12.7 (9.8)	50	4.2–11.6 (9.0)	20	4.4–10.5 (8.1)	23	5.7–11.0 (9.8)	26.2	4.2–12.7 (9.5)
E. cylindroides	15	3.5–10.0 (6.8)	43	9.8–11.9 (10.8)	7	9.7	23	3.7–11.6 (9.6)	21.3	3.5–11.9 (9.6)
E. dolichum	0		0		0		7	6.5–11.0 (8.6)	2.8	6.5–11.0 (8.6)
E. eligens	8	5.5	0		7	3.7	0		3.5	3.7–10.8 (7.9)
E. formicigenerans	0		0		0		3	8.7–11.5 (10.1)	1.4	8.7–11.5 (10.1)
E. hallii	0		7	8.4	0		5	4.4–10.6 (7.1)	2.8	4.4–10.6 (7.5)
E. lentum	15	3.6–7.9 (5.7)	36	3.6–11.6 (9.6)	53	6.0–10.8 (8.3)	47	5.4–11.4 (9.4)	42.6	3.6–11.6 (9.3)
E. limosum	0		0		13	5.8–7.0 (6.4)	7	8.7–10.8 (9.6)	5.7	5.8–10.8 (8.7)
E. moniliforme	8	8.9	21	10.6–11.1 (10.8)	7	9.8	5	8.0–9.7 (9.1)	6.4	8.0–11.1 (9.9)
E. multiforme	0		0		0		0		0.7	9.0
E. nitritogenes	0		0		7	9.5	3	7.5–8.0 (7.7)	2.8	4.0–9.5 (7.2)
E. ramulus	0		0		0		2	8.6	0.7	8.6
E. rectale	31	4.9–11.3 (9.2)	43	3.5–11.7 (8.6)	47	7.1–11.6 (9.0)	16	5.7–11.6 (9.4)	27.7	3.5–11.7 (9.3)
E. saburreum	0		0		0		2	7.9	1.4	7.9
E. siraeum	0		0		0		11	5.8–10.3 (9.1)	7.1	5.8–11.0 (9.6)
E. tenue	8	11.2	7	4.5	7	5.4	11	6.2–10.7 (9.3)	8.5	4.5–11.2 (8.8)
E. tortuosum	8	4.6	7	5.5	0		13	4.7–11.5 (8.9)	11.3	4.0–11.5 (8.5)
E. ventriosum	8	9.7	14	8.5–11.0 (9.8)	7	7.5	18	4.0–12.3 (9.2)	12.1	4.0–12.3 (9.2)
Species[g]	46	4.5–11.5 (10.0)	57	4.0–11.5 (8.4)	73	7.6–11.3 (9.9)	60	3.7–13.3 (9.3)	58.2	3.7–13.3 (9.5)
Total count of *Eubacterium*	92	8.0–12.7 (11.0)	93	9.5–12.0 (11.3)	93	9.7–11.8 (10.6)	95	5.9–13.3 (10.6)	94.0	5.0–13.3 (10.7)
Lactobacillus										
L. acidophilus	62	7.0–11.4 (9.8)	50	7.0–11.4 (10.3)	53	4.5–10.9 (8.0)	36	5.8–11.9 (9.2)	44.7	4.5–11.9 (9.2)
L. brevis	8	11.4	7	4.1	7	10.7	3	9.0–12.5 (10.7)	5.0	4.1–12.5 (8.8)
L. buchneri	0		0		0		0		1.4	5.4–9.8 (7.6)
L. casei	0		7	6.3	0		2	10.1	2.8	6.3–10.1 (8.4)
L. catenaforme	0		7	9.8	0		0		0.7	9.8
L. crispatus	8	4.9	0		0		0		0.7	4.9

L. fermentum	31	8.6–11.7 (10.3)	43	4.1–10.6 (6.7)	27	5.4–10.4 (8.8)	42	3.6–11.5 (8.3)	38.3	3.6–11.7 (8.4)
L. helveticus	8	10.1	0		0		3	3.6–4.7 (4.1)	2.1	3.6–10.1 (6.1)
L. lactis	8	10.6	0		0		3	7.8–9.6 (8.7)	5.0	6.4–10.9 (9.1)
L. leichmannii	0		21	6.3–6.5 (6.4)	7	8.6	0		3.5	5.4–8.6 (6.7)
L. minutes	8	8.3	14	10.1–10.8 (10.4)	0		7	8.3–10.6 (9.4)	5.7	8.3–10.8 (9.5)
L. plantarum	62	4.6–12.0 (10.0)	64	7.6–12.1 (9.9)	13	7.7–9.8 (8.8)	24	4.9–10.7 (8.0)	29.8	4.6–12.1 (8.8)
L. salivarius Subspecies *salicinius*	0		0		0		5	6.8–8.0 (7.2)	6.4	6.8–11.3 (8.3)
rogosae	0		0		0		2	10.8	0.7	10.8
Species[h]	23	3.5–12.0 (8.9)	14	8.6–10.6 (9.6)	20	5.5–10.0 (8.1)	7	5.7–11.5 (8.9)	13.5	3.5–12.0 (8.4)
Total count of *Lactobacillus*	85	8.6–12.1 (11.1)	100	6.4–12.1 (10.2)	73	4.5–10.9 (9.0)	73	3.6–12.5 (9.3)	78.0	3.6–12.5 (9.6)
Total count of Gram-positive non-spore-forming anaerobic rods	100	8.6–12.7 (11.7)	100	10.1–12.3 (11.6)	100	10.0–11.9 (10.8)	100	8.6–13.7 (11.1)	99.3	8.5–13.7 (11.1)

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[a] For a description of subjects, see footnote *a*, **Table 2**.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects.

[d] Two isolates (two species) could not be speciated using currently accepted identification protocols and presently recognized species.

[e] Two isolates (one species).

[f] Three isolates (three species).

[g] One hundred and five isolates (representing 55 possible separate species) could not be speciated using currently accepted identification protocols and presently recognized species.

[h] Nineteen isolates could not be speciated using currently accepted identification protocols and presently recognized species.

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 5 Clostridia recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly Vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Clostridium										
C. acetobutylicum	0		0		0		2	7.6	2.8	7.6–10.7 (8.6)
C. aminovalericum	8	11.9	14	6.3–7.9 (7.1)	13	5.8–6.2 (6.0)	13	5.7–10.6 (7.9)	14.2	5.7–11.9 (7.9)
C. aurantibutyricum	0		0		0		3	8.3	1.4	8.3
C. barati	15	4.1	7	3.5	13	6.5–7.1 (6.8)	8	5.4–8.7 (7.1)	9.9	3.5–9.1 (6.5)
C. barkeri	0		0		0		8	9.1–10.5 (9.8)	3.5	9.1–10.5 (9.8)
C. beijerinckii	0		14	4.1–10.7 (7.4)	0		8	5.6–12.7 (9.3)	6.4	4.1–12.7 (9.0)
C. bifermentans	0		0		20	4.3–8.2 (6.3)	32	3.3–12.6 (8.8)	29.1	3.3–12.6 (8.7)
C. butyricum	0		21	3.8–10.2 (6.4)	7	5.4	2	10.4	5.0	3.8–10.4 (6.8)
C. cadaveris	0		0		0		2	5.6	1.4	5.6–8.7 (7.1)
C. carnis	0		0		0		5	6.3–7.8 (7.3)	5.7	6.3–10.7 (8.9)
C. celatum	0		0		0		0		1.4	5.7–8.8 (7.2)
C. cellobioparum	0		0		0		3	5.7–6.5 (6.1)	3.5	5.7–9.7 (7.7)
C. chauvoei	0		0		7	4.7	5	5.9–11.1 (8.8)	2.8	4.7–11.1 (7.8)
C. clostridioforme	8	11.7	0		7	6.0	8	9.6–11.1 (10.3)	8.5	4.9–11.7 (9.0)
C. cochlearium	0		7	4.8	0		5	5.7–10.8 (7.7)	2.8	4.8–10.8 (7.0)
C. difficile	0		0		0		2	4.8	0.7	4.8
C. fallax	0		0		0		3	7.9–9.6 (8.7)	1.4	7.9–9.6 (8.7)
C. felsineum	0		0		7	8.7	0		0.7	8.7
C. ghoni	0		0		0		2	10.4	0.7	10.4
C. glycolicum	0		7	5.1	13	5.6–8.8 (7.2)	7	5.0–6.3 (5.5)	5.7	5.0–8.8 (6.2)
C. haemolyticum	0		0		0		3	7.3–10.8 (9.1)	1.4	7.3–10.8 (9.1)
C. indolis	0		7	10.1	0		2	11.0	1.4	10.1–11.0 (10.6)
C. innocuum	8	7.9	43	6.9–11.4 (9.9)	53	6.7–11.4 (9.2)	21	6.5–9.2 (8.2)	26.2	4.5–11.4 (8.6)
C. irregularis	0		0		0		2	7.0	0.7	7.0
C. lentoputrescens	0		0		0		2	5.7	0.7	5.7
C. limosum	0		7	5.3	0		2	4.4	1.4	4.4–5.3 (4.8)
C. malenominatum	0		0		0		2	9.8	2.1	4.6–9.8 (6.4)
C. mangenoti	0		0		0		8	3.6–10.1 (6.6)	5.0	3.6–10.7 (7.3)
C. nexile	0		7	3.9	0		2	9.7	1.4	3.9–9.7 (6.8)
C. oceanicum	0		0		0		2	7.6	0.7	7.6
C. oroticum	23	10.4–12.0 (11.0)	0		7	10.5	7	6.3–10.4 (7.5)	6.4	4.4–12.0 (8.7)

C. paraputrificum	15	4.8–7.9 (6.3)	57	5.0–11.4 (8.9)	47	6.7–11.4 (9.4)	28	4.4–11.8 (8.5)	9.9	4.4–11.8 (8.5)
C. pasteurianum	0		0		0		2	10.0	0.7	10.0
C. perfringens	0		7	4.0	73	4.0–10.0 (7.6)	55	3.8–12.5 (6.9)	41.1	3.8–12.5 (7.1)
C. plagarum	0		0		0		2	5.3	0.7	5.3
C. pseudotetanicum	0		0		7	5.1	2	4.0	2.1	4.0–5.1 (4.5)
C. putrefaciens'B'	15	4.4–6.8 (5.6)	0		0		7	4.7–10.1 (7.1)	7.8	4.4–11.0 (7.7)
C. ramosum	69	7.1–11.1 (9.4)	36	6.4–11.4 (9.3)	67	7.6–10.5 (9.1)	60	4.5–12.5 (8.9)	53.2	4.5–12.5 (9.1)
C. sartagoformum	0		7	9.9	0		5	8.3–10.9 (9.6)	5.0	7.7–10.9 (9.2)
C. septicum	0		0		0		3	6.9–7.3 (7.1)	2.1	6.9–9.8 (8.0)
C. sordellii	0		0		7	8.5	2	9.9	3.5	8.5–12.3 (10.2)
C. sphenoides	8	9.5	7	5.7	13	4.7–6.7 (5.7)	5	4.0–10.3 (7.0)	6.4	3.8–10.3 (6.8)
C. sporosphaeroides	0		0		13	4.1–10.2 (7.1)	7	5.5–10.9 (8.4)	5.0	4.1–10.9 (7.8)
C. subterminale	0		0		0		2	7.5	0.7	7.5
C. tertium	0		0		13	4.9–5.9 (5.4)	8	3.9–10.7 (7.1)	5.0	3.9–10.7 (6.6)
Species[d]	15	4.0–10.0 (7.0)	43	5.7–11.0 (8.5)	47	4.7–11.2 (8.3)	34	4.4–12.5 (8.2)	39.0	4.0–12.5 (8.3)
Total count of *clostridium*	92	4.1–12.2 (9.4)	93	4.9–11.5 (8.9)	100	7.7–11.5 (9.7)	100	6.5–13.1 (10.2)	100	3.8–13.1 (9.8)

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[a] For a description of subjects, see footnote *a*, **Table 2**.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects.

[d] Sixty-seven isolates (possibly representing 45 separate species) could not be speciated using currently accepted identification protocols and presently recognized species.

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 6 Streptococci recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Streptococcus										
S. agalactiae	0		0		7	9.0	0		0.7	9.0
S. avium	0		0		0		2	10.8	0.7	10.8
S. bovis	31	6.0–7.6 (6.6)	14	7.2–8.7 (7.9)	27	4.7–10.9 (7.9)	19	4.2–11.5 (7.3)	18.4	4.2–11.5 (7.5)
S. cremoris	8	7.6	14	5.4–8.2 (6.8)	0		8	5.3–10.7 (8.4)	7.1	4.6–10.7 (7.6)
S. durans	8	6.9	21	4.7–7.0 (6.1)	7	8.0	11	4.7–12.7 (7.5)	10.6	4.7–12.7 (7.0)
S. equinus	0		0		7	7.9	3	9.2–10.6 (9.9)	2.1	7.9–10.6 (9.2)
S. equisimilis	0		7	8.3	0		5	4.9–9.3 (7.1)	5.0	4.9–9.9 (7.2)
S. faecalis group	69	4.5–10.6 (7.5)	79	4.7–11.1 (7.5)	93	4.4–10.6 (7.9)	82	3.6–10.9 (7.4)	80.1	3.6–11.2 (7.5)
S. faecium group	31	4.2–7.6 (6.3)	14	5.4–6.0 (5.7)	20	6.8–10.4 (8.6)	36	3.5–10.9 (8.0)	30.5	3.5–11.5 (7.9)
Group G	0		0		0		2	6.5	0.7	6.5
Group M	8	8.0	0		0		0		0.7	8.0
Group O	0		0		0		0		1.4	6.4–8.6 (7.5)
S. lactis	31	6.2–11.8 (8.4)	29	5.5–8.1 (6.6)	7	8.2	29	5.5–12.3 (8.7)	28.4	5.5–12.3 (8.2)
MG-*intermedius*	15	7.6–8.8 (8.2)	7	6.3	13	6.0–8.7 (7.3)	0		3.5	6.0–8.8 (7.5)
S. mitis	15	4.0–7.9 (5.9)	21	6.6–10.6 (8.5)	20	6.4–7.1 (6.7)	34	5.1–10.7 (7.9)	31.2	4.0–10.7 (7.3)
S. mutans	8	7.3	7	8.7	0		11	5.6–9.9 (7.7)	11.3	5.6–9.9 (7.4)
S. pyogenes	0		0		0		2	7.5	0.7	7.5
S. salivarius	15	4.7–6.0 (5.4)	14	5.5–7.7 (6.6)	0		10	4.1–9.4 (6.9)	12.1	4.1–9.8 (7.1)
S. sanguis	39	4.6–10.2 (7.0)	29	3.7–7.0 (5.0)	7	4.1	13	5.2–9.4 (7.4)	16.3	3.7–10.2 (6.7)
S. thermophilus	0		0		0		2	7.6	0.7	7.6
S. uberis	0		0		7	9.4	0		0.7	9.4
S. zooepidemicus	0		0		0		2	5.6	0.7	5.6
Species[d]	62	5.9–11.0 (8.2)	57	6.2–11.1 (7.9)	60	5.1–10.9 (8.3)	53	4.4–11.0 (8.4)	51.8	4.4–11.1 (8.2)
Total count of *Streptococcus*	100	6.0–11.8 (8.6)	100	5.8–11.1 (8.7)	100	5.2–11.1 (8.7)	100	5.1–12.9 (9.1)	99.3	3.9–12.9 (8.9)

[a] For a description of subjects, see footnote *a*, **Table 2**.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects.

[d] Seventy-three isolates could not be speciated using currently accepted identification protocols and presently recognized species.

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 7 Other facultative organisms recovered from patients in V.A. Wadsworth Medical Center Fecal Flora Studies[a,b]

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Gram-negative facultative bacilli										
***Aeromonas** hydrophila*	0		0		0		0		2.1	2.7–9.5 (7.0)
Alcaligenes species	8	7.3	0		0		0		0.7	7.3
***Citrobacter** freundii*	15	6.4–7.8 (7.1)	7	6.1	7	3.7	2	9.5	4	3.7–9.5 (7.0)
Citrobacter species	0		7	7.5	27	5.6–8.6 (6.7)	10	6.3–9.7 (7.5)	9.2	5.6–9.7 (7.5)
***Enterobacter** aerogenes*	8	7.0	0		0		2	7.5	2.1	7.0–7.5 (7.3)
E. cloacae	46	4.5–9.4 (7.3)	29	3.6–7.2 (6.0)	0		13	4.3–11.5 (7.5)	16.3	3.6–11.5 (7.3)
E. liquefaciens	0		0		33	6.7–9.4 (7.6)	11	4.2–9.2 (6.5)	8.5	4.2–9.4 (6.9)
Enterobacter species	0		0		0		3	6.6–8.3 (7.5)	1.4	6.6–8.3 (7.5)
***Escherichia** coli*	92	5.9–10.9 (7.7)	86	3.9–10.7 (7.1)	100	6.3–10.8 (9.0)	94	4.4–12.3 (8.8)	92.9	3.9–12.3 (8.6)
E. coli(lactose-negative)	0		0		7	7.5	3	7.6–8.2 (7.9)	2.1	7.5–8.2 (7.8)
Hafnia species	0		7	3.9	7	8.3	2	5.6	2.1	3.9–8.3 (6.0)
***Klebsiella** ozaenae*	8	5.6	0		0		2	6.7	1.4	5.6–6.7 (6.2)
K. pneumoniae	46	4.5–11.7 (8.4)	43	3.7–11.6 (6.9)	20	7.0–9.8 (8.1)	15	4.4–10.0 (7.6)	19.9	3.7–11.7 (7.7)
Klebsiella species	0		0		33	5.3–9.3 (7.6)	24	3.5–10.1 (6.9)	19.9	3.5–10.1 (7.3)
***Morganella** morganii*	0		0		7	8.3	2	4.3	1.4	4.3–8.3 (6.3)
***Proteus** mirabilis*	0		7	7.9	20	5.3–8.0 (6.9)	5	5.6–8.8 (7.3)	6.4	5.3–10.0 (7.7)
P. vulgaris	0		0		0		2	7.0	0.7	7.0
Providencia rettgeri	0		0		0		1	10.6	0.7	10.6
***Pseudomonas** aeruginosa*	23	4.3–6.9 (5.4)	0		20	2.9–5.0 (3.8)	5	4.5–7.3 (5.5)	10.6	2.9–8.7 (5.0)
Pseudomonas species	8	3.4	0		0		0		1.4	3.4–8.7 (6.1)
Total count of Gram-negative facultative anaerobes	100	6.3–11.7 (8.2)	100	3.9–11.6 (7.4)	100	6.8–10.8 (9.2)	98	4.9–12.4 (8.9)	98	4.0–12.4 (8.7)
Other facultative organisms										
***Aerococcus** viridans*	8	7.4	0		0		0		0.7	7.4
Bacillius species	69	3.5–5.0 (4.2)	86	3.9–5.5 (4.4)	80	3.9–10.0 (6.2)	82	0.6–9.9 (5.0)	82.3	0.7–10.9 (5.2)
Corynebacterium species	8	7.6	7	6.6	0		2	7.6	2.1	6.6–7.6 (7.3)
Micrococcus species	0		14	4.3–5.8 (5.1)	7	8.5	11	3.7–9.3 (6.4)	10.6	3.7–10.6(7.2)

(Continued)

Table 7 (Continued)

	Strictly vegetarian (13)[c]		*Vegetarian, some meat (14)*		*Japanese diet (15)*		*Western diet (62)*		*Total (141)*	
Organism	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*	*% positive*	*Range (mean)*
Nocardia species	8	7.4	0		20	6.6–8.4 (7.7)	0		2.8	6.6–8.4 (7.7)
Pediococcus species	0		0		0		2	7.7	0.7	7.7
Staphylococcus *aureus*	23	4.2–7.9 (5.5)	7	4.0	13	3.7–7.6 (5.7)	11	3.6–6.4 (4.6)	11.3	3.6–8.9 (5.4)
Staphylococcus epidermidis	46	4.0–8.8 (5.9)	36	4.1–11.4 (8.0)	20	3.7–9.8 (5.9)	27	3.9–12.7 (8.2)	31.2	3.7–12.7 (7.4)
Candida *albicans*	15	3.5–6.3 (4.9)	0		47	3.5–8.9 (5.6)	14	3.6–9.4 (5.4)	14.2	3.5–9.4 (5.4)
Candida species	0		0		13	3.7–8.8 (6.3)	8	3.7–5.1 (4.4)	5.0	3.7–8.8 (4.9)
Other yeast	23	4.3–7.8 (5.6)	50	4.2–8.7 (6.1)	53	3.4–8.7 (5.8)	31	3.6–8.1 (5.2)	36.2	3.4–8.7 (5.6)
Filamentous fungi	0		0		0		3	3.5–4.0 (3.8)	3.5	3.5–8.0 (5.9)
Total count of other facultative organisms	85	3.9–8.8 (5.8)	100	3.9–11.4 (6.5)	93	5.1–10.3 (7.3)	97	0.7–12.7 (6.5)	92.9	0.7–12.7 (6.8)

[a] For a description of subjects, see footnote *a*, **Table 2**.

[b] Ranges and mean counts of bacteria expressed as number of organisms $\log_{10}$ per gram feces (dry weight).

[c] Numbers in parentheses are numbers of subjects.

Reproduced from Finegold SM, Sutter VL, and Mathisen GE (1983) Normal indigenous intestinal flora. In: Hentges DJ (ed.) Human Intestinal Microflora in Health and Disease, pp. 3–31. Paris: Academic Press.

Table 8 The 25 most prevalent bacterial species in the feces of human subjects consuming a Western diet (10^9–10^{10} bacteria per gram dry weight)

1	*Bacteroides vulgatus*
2	*Bacteroides* species, other
3	*Bacteroides fragilis*
4	*Bacteroides thetaiotaomicron*
5	*Peptostreptococcus micros*
6	*Bacillus* species (all)
7	*Bifidobacterium adolescentis* D
8	*Eubacterium aerofaciens*
9	*Bifidobacterium infantis*, other
10	*Ruminococcus albus*
11	*Bacteroides distasonis*
12	*Peptostreptococcus intermedius*
13	*Peptostreptococcus* sp.2
14	*Peptostreptococcus productus*
15	*Eubacterium lentum*
16	Facultative streptococci, other
17	*Fusobacterium russii*
18	*Bifidobacterium adolescentis* A
19	*Bifidobacterium adolescentis* C
20	*Clostridium clostridiiformis*
21	*Peptostreptococcus prevotii*
22	*Bifidobacterium infantis* ss. *liberorum*
23	*Clostridium indolis*
24	*Enterococcus faecium*
25	*Bifidobacterium longum* ss. *longum*

Modified from Tannock GW (1995) Normal microflora. Modified from Finegold, *et al.* (1974) Effect of diet on human fecal flora; comparison of Japanese and American diets. *American Journal of Clinical Nutrition* 27: 1456–1469 with permission.

API ZYM kit are available) has been found to be very useful. More importantly, quite a large number of genera and species are newly described so that studies such as those described above need to be updated; at this time, sequencing of 16S rDNA and other genes plus use of expanded phenotypic testing schemes would be used to provide a much more detailed and accurate picture. There are studies that have reanalyzed old taxa and provided important new information. For example, the organism *Clostridium clostridioforme* is now known to be comprised of a group of related species – *C. bolteae, C. hathewayi, C. clostridioforme* (newly defined), *C. asparagiforme, C. citroniae* and *C. aldenense.* The genus *Bacteroides* has been modified in several ways. *B. distasonis, B. merdae*, and *B. goldsteinii* have been placed in a new genus, *Parabacteroides* and a new species *Parabacteroides johnsonii* has been added. *Bacteroides putredinis* has been moved into a new genus, *Alistipes* and three new species have been added to it: *A. finegoldii, A. onderdonkii*, and *A. shahii.* There are many other newly described taxa as well. New species of *Bacteroides* are *B. coprocola, B. plebeius, B. finegoldii, B. massiliensis, B. nordii, B. salyersiae*, and *B. doreii. Fusobacterium prausnitzii* has been moved to a new genus *Faecalibacterium* as *F. prausnitzii.* There are two new species of *Porphyromonas* that may be found in the gut – *P. uenonis* and *P. levii.* Newly described clostridial species include *C. bartlettii, C. neonatale*, and *C. disporicum.* The Gram-positive nonsporeforming bacilli have many new taxa described. Among the *Actinobacteria* are the genera *Actinobaculum* and *Collinsella* and among the *Firmicutes* are the genera *Anaerofustis, Anaerotruncus, Anaerostipes, Holdemania*, and *Solobacterium.* The taxonomy of the anaerobic cocci has been completely revised, with several new genera added – *Anaerococcus, Parvimonas, Peptoniphilus, Finegoldia*, and *Gallicola.* There are also new species of *Ruminococcus.* Certain strains of *Bacteroides fragilis* are now known to produce an enterotoxin that has been found to produce diarrhea in certain populations.

Molecular Studies of the Microflora of the Colon

Molecular studies of colonic flora give a better picture of the true flora than cultural studies. Molecular identification of isolates also gives greater accuracy and speed than phenotypic identification. Both types of studies, used together, give more accurate identification of certain taxa and studies of additional genes (beyond 16S rDNA) are needed for certain organisms (viz., streptococci and staphylococci). Various molecular approaches to the study of the colonic microflora have been used. Included are denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis; when these approaches are combined with cloning and sequencing, the results are improved. Group-specific primers have been used for detection and identification of predominant groups of bacteria in feces. This approach, combined with FISH, is effective but only small portions of the total bacteria have been detected in some fecal samples in some studies. The study of emergent bowel resection, mentioned above in the section on ileal flora, found that there were no significant differences in overall numbers of bacteria in different parts of the colon. Real-time PCR showed that bifidobacteria counts were significantly higher in the large bowel than in the terminal ileum, *Eubacterium rectale* and *F. prausnitzii* were dominant in the ascending and descending colons, and lactobacilli were more prominent in the distal large bowel. Benno's group has had good results with T-RFLP studies of bowel flora, using a data base that they have developed for this purpose. The use of group-specific probes with real-time PCR is a very good technique which provides a good snapshot of the bowel flora with quantitation if one uses an extensive set of appropriate primer-probes. This approach has been used to compare the microbiota of different groups of individuals such as infants and elderly people with the general population. Detection limits with real-time PCR are as low as 10^1 or fewer organisms of a particular phylotype per sample. With the newly available real-time PCR equipment, extremely high throughput is now available; included are trays with 384 wells so that if one develops all the appropriate primer-probes, it is now feasible to study a large number of individual genera and/or species rather than groups or clusters of organisms. DNA microarray analysis cannot be used effectively by most smaller laboratories unless reliable commercially available kits are available but one study showed good results with a microarray of 40 species commonly encountered in the gut. A problem with several of these techniques such as FISH, real-time PCR, and microarrays is that one can only find the organisms or groups that one has specific primer-probes for and there is still a considerable portion of the bowel flora that remains uncharacterized.

A tedious and time-consuming study of clone libraries, and one not suitable for high throughput at this time, is nonetheless an excellent procedure for detailed analysis of colonic or other diverse and complicated microfloras. Newly available pyrosequencing machines, when fully developed, will greatly assist in this type of analysis. The clone library approach has been used effectively by a number of investigators and has provided outstanding data from the study of small numbers of individuals. For this procedure, DNA is recovered from the sample to be studied, it is enriched and amplified for the 16S rDNA using broad-range primers, and then the PCR products are cloned into *Escherichia coli* by established procedures. The 16S rDNA nucleotide sequences of the clone inserts are determined by cycle sequencing and trimmed to remove vector sequence, chimeras, and sequences of poor quality. Sequences are grouped into phylotypes so that the least similar pair within the phylotype has 99% similarity (some use 98% as the cutoff). In an elegant study of the diversity of the human intestinal flora, both fecal samples and mucosal biopsies from six different colonic sites were obtained from three healthy adults. In all, these workers performed phylogenetic analyses on a total of 11 831 bacterial and 1524 archaeal near-full-length 16S rDNA sequences (**Figures 2** and **3**). From this, they identified 395 bacterial phylotypes and a single archaeal phylotype (*Methanobrevibacter smithii*). Most of the bacteria were members of the *Firmicutes* and *Bacteroidetes* phyla. Within the *Firmicutes* phylum, there were 301 phylotypes, 191 of which were novel; 95% of the *Firmicutes* sequences were members of the class *Clostridia*. Known butyrate-producing bacteria represented 2454 sequences and 42 phylotypes, all belonging to clostridial clusters IV, XIVa, and XVI. There were 65

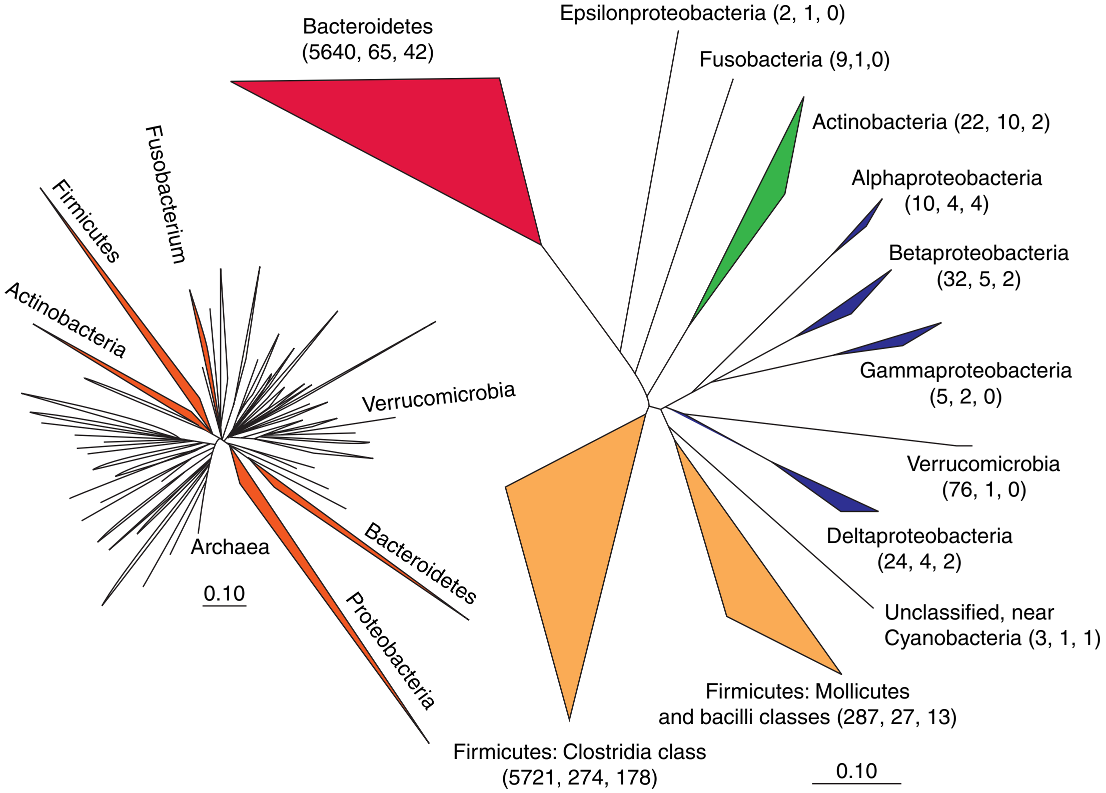
Bacteroidetes
(5640, 65, 42)
Epsilonproteobacteria (2, 1, 0)
Fusobacteria (9,1,0)
Actinobacteria (22, 10, 2)
Alphaproteobacteria
(10, 4, 4)
Betaproteobacteria
(32, 5, 2)
Gammaproteobacteria
(5, 2, 0)
Verrucomicrobia
(76, 1, 0)
Deltaproteobacteria
(24, 4, 2)
Unclassified, near
Cyanobacteria (3, 1, 1)
Firmicutes: Mollicutes
and bacilli classes (287, 27, 13)
Firmicutes: Clostridia class
(5721, 274, 178)
0.10
Fusobacterium
Firmicutes
Actinobacteria
Verrucomicrobia
Archaea
0.10
Bacteroidetes
Proteobacteria

Figure 2 Phylogenetic tree based on the combined human intestinal 16S rDNA sequence data set. The label for each clade includes, in order, the total number of recovered sequences, phylotypes, and novel phylotypes (in parentheses). The angle where each triangle joins the tree represents the relative abundance of sequences, and the lengths of the two adjacent sides indicate the range of branching depths within that clade. Six of the seven phyla represented by sequences recovered in this study are shown in red; the unclassified clade near cyanobacteria is not pictured in the inset. Reproduced with permission from Eckburg PB, Bik EM, Bernstein CN, *et al*. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.

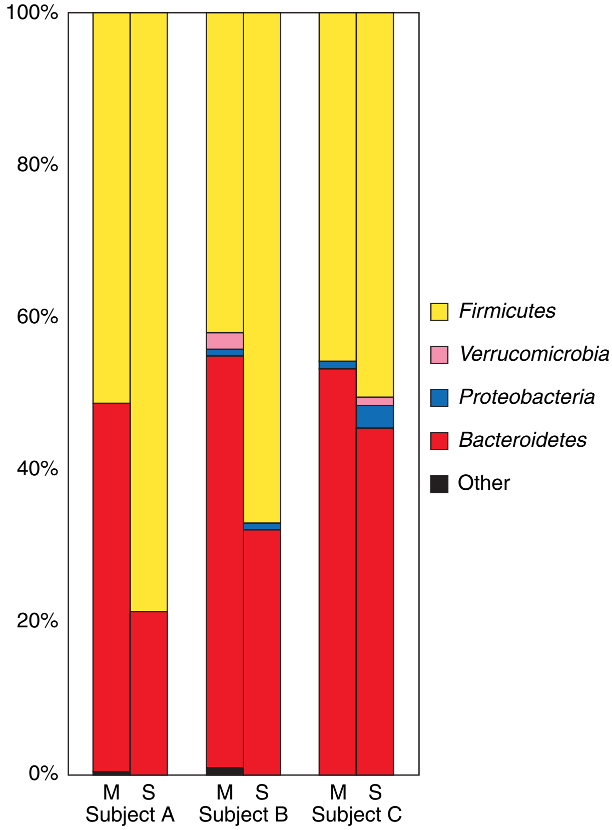

Figure 3 Relative abundance of sequences from stool and pooled mucosal samples per subject. The sequence frequencies are grouped according to phylum, colored according to **Figure 2**. 'Other' represents the fusobacteria, actinobacteria, and unclassified near cyanobacteria phyla, each containing less than 0.2% of the total sequences. 'M' denotes pooled mucosal sequences per subject and 'S' refers to stool sample. Reproduced with permission from Eckburg PB, Bik EM, Bernstein CN, *et al*. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.

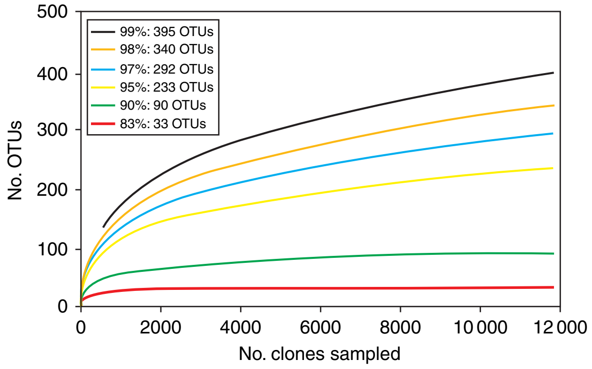

Figure 4 Individual-based rarefaction curves for combined sequences at multiple operational taxonomic unit (OTU) cutoff levels. The slopes of the curves decrease as the OTU definitions relax toward 95%. The curves seem to plateau at OTU cutoffs 90%; for example, at the 90% cutoff, the last 430 clones sampled do not change the final richness value of 90 phylotypes. Every clone sampled has been seen more than once at the 83% OTU cutoff. The total numbers of OTUs per definition are listed in the inset, as calculated by dissimilarity matrices and DOTUR. Reproduced with permission from Eckburg PB, Bik EM, Bernstein CN, *et al*. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.

Bacteroidetes phylotypes, with large variations between the three study subjects. *Bacteroides thetaiotaomicron* was present in all three individuals. Relatively few sequences were associated with the *Proteobacteria, Actinobacteria, Fusobacteria*, and *Verrucomicrobia* phyla. Both the observed and estimated richness of the flora increased in parallel fashion with additional sampling; the authors estimate that one unique phylotype would be expected for every 100 additional clones sequenced (**Figure 4**). There was relatively little variability between the six mucosal sites studied. The greatest amount of variability overall was related to differences between the three subjects whose specimens were analyzed (**Figure 5**) with the next greatest variability related to differences between feces and mucosal analyses. Overall, a majority of the bacterial sequences encountered belonged to uncultivated species and novel bacteria. The authors of this study point out that the limited sensitivity of broad-range PCR may hinder detection of rare phylotypes and that their methods did not distinguish between living and dead microorganisms. Studies by various groups proceeded beyond the above approach to analyze the metagenomics (metabolic function analysis) of the bowel flora.

One group recently proposed a novel approach for comparing 16S rRNA gene clone libraries that is independent of both DNA sequence alignment and definition of bacterial phylogroups; they used direct comparisons of microbial communities from the human GI tract in an absolute evolutionary coordinate space.

Studies of Individual or Special Groups

Various groups have used a variety of methods to detect and sometimes quantitate special populations such as sulfate-reducing bacteria (*Desulfovibrio* specifically), *Bifidobacterium*, lactobacilli, *Methanobrevibacter smithii*, various clostridia, and a mucin-degrading bacterium known as *Akkermansia muciniphila*. Fecal flora studies have also been undertaken to study the relationship between intestinal bacteria (particularly bifidobacteria and lactobacilli) and aging, to note changes in bowel flora following administration of certain antimicrobial agents, to study microflora changes in relation to administration of lactulose and *Saccharomyces boulardii*, to study the GI tract microflora in certain diseases such as inflammatory bowel disease, *Clostridium difficile*-associated colitis or diarrhea, and autism, in comparison with control subjects, and to note flora involved in lactate formation and

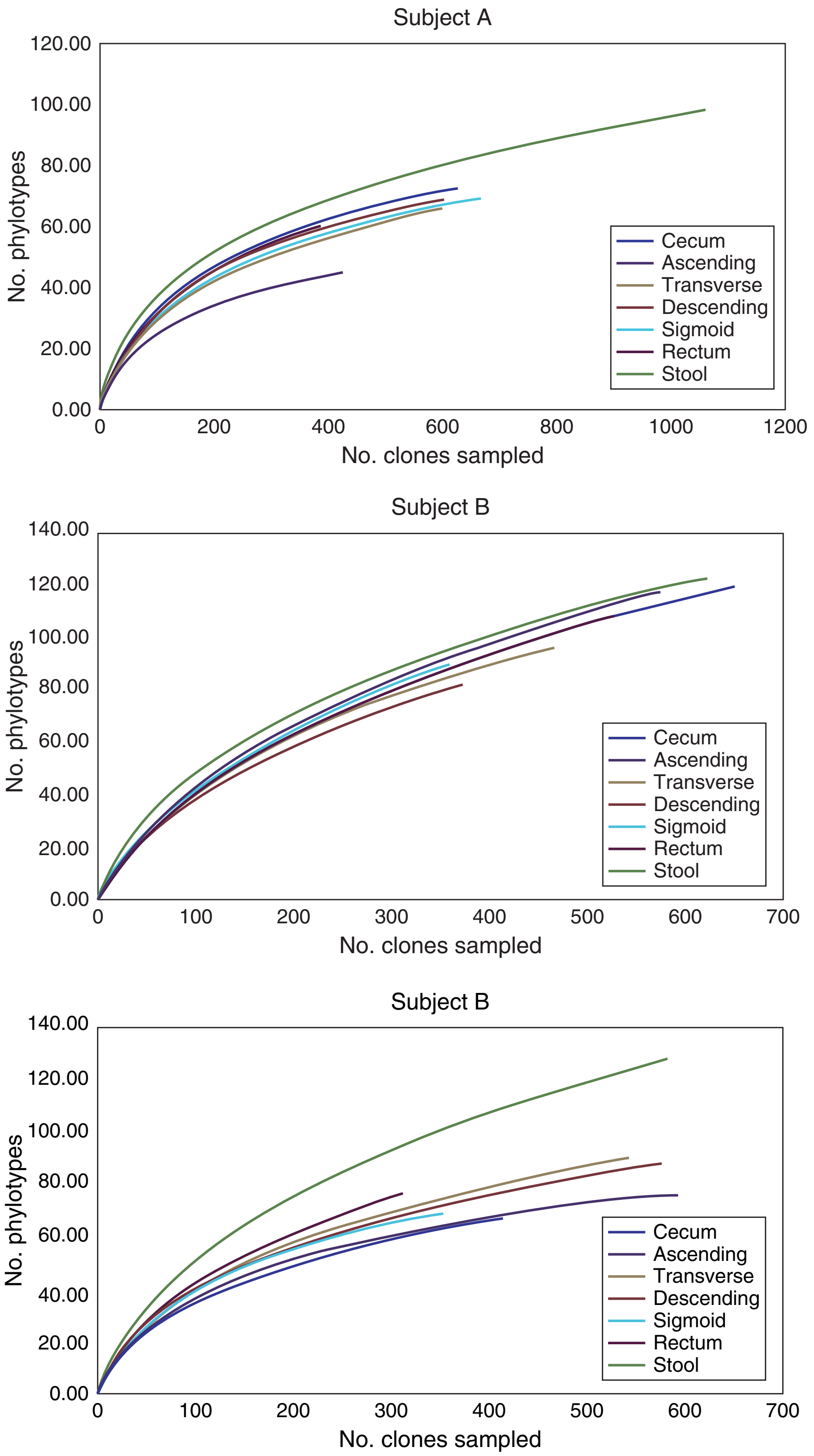

Figure 5 Individual-based rarefaction curves for sequences from each anatomic site per subject. Phylotypes were defined using the 99% operational taxonomic unit (OTU) cutoff. Reproduced with permission from Eckburg PB, Bik EM, Bernstein CN, *et al*. (2005) Diversity of the human intestinal microbial flora. *Science* 308: 1635–1638.

conversion to short-chain fatty acids. A fascinating paper on the symbiotic relationships between the human host and its GI tract microflora has been published. Their studies involved models of germ-free mice colonized with specific human microflora and comparisons of genomes of members of the bowel flora. One striking example of this research is documentation of the importance of *Bacteroides thetaiotaomicron* for the host and its highly developed environmental sensing apparatus and its capacity for retrieving polysaccharides from the gut lumen. These workers point out that polysaccharides are the most abundant biological polymer on Earth and that polysaccharide fermentation is an important activity in bacterial communities and contributes to ecologically important processes, including the recycling of carbon. Follow-up publications have attracted a great deal of interest; they describe an obesity-associated GI tract microbiome with a transmissible trait such that colonization of germ-free mice with an obese microbiota leads to significant increase in total body fat. Genetically obese mice have fewer *Bacteroidetes* and correspondingly more *Firmicutes*. Studies on 12 obese people showed similar proportions of these phyla and when they were placed on either a fat-restricted or a carbohydrate-restricted low calorie diet for 1 year showed an increase in *Bacteroidetes* and a decrease in *Firmicutes*, regardless of diet type. Remarkably, these changes were division-wide and not related to specific bacterial species. It has been pointed out that, in relation to dietary advice, consideration needs to be given to the role of carbohydrates in maintenance of gut health and function. Microbial fermentation releases as much as 10% of dietary energy in the form of short-chain fatty acids that act as a source of energy for host cells. Butyrate is a preferred energy source for colonic epithelial cells and has been implicated in the prevention of colitis and colorectal cancer. Counts of *Roseburia* spp. and *Eubacterium rectale*, and bifidobacteria to a lesser extent, decrease as carbohydrate intake decreases. This correlates with a decline in fecal butyrate.

Addendum

The use of pyrosequencing for evaluating diversity has been improved remarkably in the past several months. One can use a modified tag-encoded bacterial diversity amplificon method, which promotes studies of human microbiomes through the increased number of individual samples ($n > 200$), that can be included as part of a single massively parallel FLX pyrosequencing reaction. Segments of 250 bp can now be achieved and it is likely that this will be increased to 500 bp by the end of 2008. Rarefaction techniques can be utilized to detect organisms in counts of $>10^3\,g^{-1}$ in fecal specimens with a total count of $10^{14}\,g^{-1}$.

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Genome Sequence Databases: Genomic, Construction of Libraries

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Defining Statement
Vectors Used in Genomic Library Construction
Genomic DNA Preparation
Agarose Gel Electrophoresis Used in Genomic Library Construction
Quantifying DNA and Determining Quality
Ligation Reactions
Transformation of Library DNA into Bacterial Host Strains
Increasing the Number of Transformants
Determining the Number of Transformants Needed for Coverage of an Entire Genome
Current Strategies to Enhance Genomic Library Production
Sample Protocol to Construct a 4 kb *Pseudomonas aeruginosa* PAO1 Library with Vector PBTB-1 Within an *E. coli* Host Strain
Further Reading

Glossary

agarose gel electrophoresis Movement of charged molecules, such as proteins and nucleic acids, induced by an electrical field.

blunt ends DNA fragment ends with no overhanging nucleotides resulting from enzyme digestions.

cohesive ends DNA fragment ends with single-stranded overhangs resulting from enzyme digestions; also known as sticky ends.

competent cell Cells treated to accept extracellular DNA.

copy number Number of particular plasmids present in a cell.

electroporation The process of introducing vectors into a host organism by temporarily creating electropores to allow DNA passage.

end repair Process employing Klenow fragments or DNA polymerases to fill in or remove overhangs obtained by restriction enzyme digestions.

genomic library Collection of overlapping segments of DNA including all regions of an organism's genome.

ligase Enzyme catalyzing the reformation of phosphodiester bonds to join two compatible DNA fragments.

ligation Joining of two compatible fragments of DNA through the reformation of phosphodiester bonds catalyzed by ligase.

partial digestion Enzyme digestion performed for a limited amount of time that does not go to completion for purposes of generating random DNA fragments for library construction.

plasmid Extrachromosomal, circular DNA molecule employed to introduce DNA fragments into a host organism.

recombinant clone Clone containing a recombinant DNA molecule.

restriction enzyme Enzyme recognizing a specific DNA sequence around which it will cleave both strands of a DNA segment.

transformation Introduction of foreign DNA into a host organism.

vector DNA molecule, such as a plasmid, into which exogenous DNA fragments can be ligated for transformation into a host organism and propagation within that host.

Abbreviations

CFU	colony forming units
LB broth	Luria–Bertani broth

Defining Statement

Genomic libraries are becoming more important as the uses for biotechnology multiply and expand at an increasing pace. Identifying and isolating genes of interest, as well as gene mapping and sequencing, must be preceded by construction of a genomic library. The quality and specific features of such a library are therefore of utmost importance.

Vectors Used in Genomic Library Construction

Vector Selection

The choice of backbone vector used for constructing a genomic library is highly dependent upon what studies will be performed. When choosing a backbone vector, decisions must be made about the desired copy number, selective marker, size of genomic DNA insert, host range, and, if the cloned DNA is to be expressed, the type of promoter and ribosomal binding site that should be upstream of the multiple cloning site. Also, the vector used for library construction should remain stable after genomic DNA fragments are inserted.

For constructing a genomic library, it is important that the vector has the ability to replicate within the desired host organism or organisms. The origin of replication, or the *ori* region, on a vector controls the host range and, to a large extent, the copy number of the vector. Vectors with narrow host ranges such as those containing the ColE1 origin have only been found to replicate in *Escherichia coli* and closely related bacteria. Broad host range vectors have origins of replication that are recognized in a wide range of bacterial species. The origin of replication from the broad host range plasmids RK2 and pBBR1 is functional across multiple Gram-negative species while plasmid RSF1010 has been found to be able to replicate within a number of both Gram-positive and Gram-negative species.

The *ori* region (replicon) of a vector also affects the copy number of vector within the host. High copy number vectors offer the advantage of increased yield of purified vector from a volume of culture to be used for sequencing or molecular cloning purposes. Low copy number vectors are typically desired for studies involving expression of genomic DNA segments, especially those involving toxic DNA segments. When a higher yield of a low copy number vector is needed, low copy number vectors containing the ColE1 or pMB1 origin of replication, which allows for plasmid number amplification prior to purification in the presence of $170\,\mathrm{mg\,l^{-1}}$ of chloramphenicol, may be used. Chloramphenicol inhibits protein synthesis and thus prevents chromosomal replication. The enzymes necessary for replication of plasmids with the ColE1 or pMB1 origin require enzymes that are long lived and thus continue to replicate in the presence of chloramphenicol, reaching several thousand copies per cell.

The *ori* region and the mechanism of vector replication have also been implicated to be responsible for the structural stability of cloned vectors. Vectors with replicons using rolling circle mechanisms have frequently been found to be unstable for cloning purposes. This instability may be due to the secondary structure formed by the lagging strand of DNA during replication, which may be cleaved by nucleases or experience mutations or deletions during replication.

Features of and around the insert site also have an impact on stability and representation of the library. For the creation of a representative genomic library, all DNA sections of the genome must be cloned, regardless of DNA secondary structure or the encoding of toxic gene products or strong promoters. Vectors that contain transcription terminators flank the cloning site to prevent transcription into and out of the cloned DNA and have been shown to be successful at mitigating cloning bias against difficult DNA and increase vector stability. For studies involving expression of the insert DNA, a variety of vectors exist that offer options of using constitutive, inducible, titratable, or native promoters, as well as options including ribosomal binding sites and start codons upstream of the cloned insert.

Vector Preparation

Once an appropriate vector has been selected, it must be prepared for cloning. Purified vector should be free of endonuclease contamination and chemicals such as phenol or EDTA that may interfere with downstream enzymatic reactions. There exist a number of standard protocols and commercially available kits that are used for purifying plasmid DNA. Plasmid purification protocols take advantage of the smaller size of the plasmid DNA compared to the significantly larger chromosomal DNA. The method chosen is influenced in part by the size of the plasmid and the host strain. Many commonly used cloning strains, such as *E. coli* DH5α, have mutations within their *endA* regions, which eliminate the nonspecific endonuclease activity of endonuclease I, thus allowing higher quality plasmid preparations from these strains. Other strains such as *E. coli* HB101 produce large amounts of carbohydrates that can interfere with DNA extractions. Large plasmids (>15 kb) are more fragile and thus have to be treated with more gentle extraction methods than small plasmids, which are less susceptible to damage.

Once purified, a vector needs to be linearized prior to ligation. Using the sequence of the vector and the vector map, an enzyme or a set of enzymes must be found that cut only within the cloning site of the vector. Enzymes may leave blunt or sticky ends after digestion. When selecting enzymes to digest the vector, it must be kept in mind that the ends for the vector and the ends for the fragmented genomic DNA that will be cloned must be compatible. The sticky ends of a digested piece of DNA may be converted to blunt ends by using particular DNA modifying enzymes that may fill in overhangs of sticky ends or exhibit exonuclease activity against single-stranded DNA and thus degrade overhangs. T4 DNA polymerase or the Klenow fragment of *E. coli* DNA polymerase I will remove $3'$ overhangs and fill in $5'$ overhangs when provided with deoxynucloside triphosphates. Additionally, DNA end repair kits are available from several commercial vendors that use proprietary enzymes to generate blunt-ended DNA segments.

In an effort to prevent self-ligation and minimize the number of clones without genomic DNA insert, the 5′-termini phosphate groups required by ligase for ligation reactions are removed from the linearized vector using an alkaline phosphatase. The most commonly used alkaline phosphatases within molecular biology are shrimp alkaline phosphatase from the Arctic shrimp *Pandalus borealis*, calf intestinal alkaline phosphatase from calf intestines, bacterial alkaline phosphatase from *E. coli* C4, and Antarctic Phosphatase from the psychrophilic bacterium strain TAB5. All of these phosphatases are effective at removing 5′ phosphates from DNA, but vary in their activity, buffer compatibility, and ability to be inactivated. Alkaline phosphatases bind tightly to DNA and thus may require aggressive methods to denature. To inactivate bacterial alkaline phosphatase and calf intestinal alkaline phosphatase reactions, proteinase K is used to digest the phosphatase, followed by a phenol–chloroform extraction and an ethanol precipitation. Alternatively, a commercial enzymatic reaction clean-up kit can be used to purify the dephosphorylated DNA. Shrimp alkaline phosphatase and Antarctic Phosphatase from TAB5 are thermolabile and can be completely heat inactivated. It desired to use an alkaline phosphatase that is compatible with the restriction enzyme buffer or buffers that were used in upstream preparation of the vector and to have an alkaline phosphatase that is heat-labile to minimize the number of purification steps, which may decrease the yield of vector DNA.

The dephosphorylation step, which removes 5′ phosphate groups from the linearized vector, may not go to completion and thus the amount of background from vector that can self-ligate may be significantly high. An optional step to help mitigate this problem is to perform a ligation reaction following the dephosphorylation step. Vector DNA that maintained the 5′ phosphate groups will self-ligate while linearized vector DNA that has had its 5′ phosphates successfully removed will be incapable of self-ligating and thus remain linear. The circular self-ligated vector DNA and the linear vector DNA can then be separated by agarose gel electrophoresis. The linearized vector can be extracted with a scalpel and purified using traditional DNA extraction methods or commercially available kits for DNA extraction from agarose gels.

If the ligation step to remove vectors that had not been successfully dephosphorylated is omitted, the linearized vector should still be purified from the restriction enzyme digestion and the alkaline phosphatase reaction buffers and enzymes. Phenol–chloroform purification followed by an ethanol precipitation or a commercially available spin column for cleanup of enzymatic reactions may be used for this purpose. Purifying the vector DNA will remove proteins and buffer components that may lessen the efficiency of the ligation of the vector to genomic DNA insert.

PCR amplification may also be used to generate large amounts of linear dephosphorylated vector. Culture-purified and linearized vector is PCR amplified with unphosphorylated primers designed to extend outward from the cloning site into the vector backbone. Proofreading polymerases such as *Pfu* or *Pfx* DNA polymerase will generate high-fidelity blunt-ended PCR product. The choice of polymerase influences the types of overhangs that will be generated as well as the fidelity of the PCR product generated. The PCR product should be purified away from the components in the PCR buffer prior to ligation so as not to have extraneous nucleotides, primers, or salts that may interfere with enzymatic reactions. To do so, the sample may be separated via agarose gel electrophoresis followed by excision of the appropriate band containing the PCR product of linearized vector. DNA can then be extracted via a commercial gel extraction kit.

Genomic DNA Preparation

Genomic DNA Purification

Genomic DNA must be isolated from proteins and other cellular debris prior to any enzymatic or mechanical manipulation. Bacterial cells are lysed, typically through exposure to surfactants, such as sodium dodocyl sulfate or Tween-20, or treatment with lysozyme to digest the polysaccharide component of cellular membranes and proteinase K for protease digestion. DNase-free RNase may be added to the lysis step to minimize RNA contamination. Genomic DNA can be purified from cell lysate using a phenol–chloroform extraction followed by an ethanol precipitation or commercially available silica columns. Commercially available kits for genomic DNA isolation are often desirable in that they avoid the use of phenol and chloroform, which are toxic and may interfere with downstream enzymatic reactions. Many commercially available kits avoid phenol by using buffers containing the chaotropic agent guanidine hydrochloride to aid in cell lysis and to effectively denature proteins. Purified genomic DNA should be maintained in a nuclease-free Tris buffer or in nuclease-free water.

Nuclease contamination is a frequent concern associated with genomic DNA isolation. Nuclease activity will degrade DNA and can be easily mistaken for a restriction enzyme digestion or the result of mechanical shearing. Nuclease contamination may be detected by incubating an aliquot of purified DNA at 37 °C for 18 h and then visualizing the DNA on an agarose gel. A control aliquot of DNA that had been stored frozen should be used for comparison. Following electrophoresis, if the incubated aliquot appears to have degraded, nucleases may be contaminating the genomic DNA sample. Additionally, the DNaseAlert kit available from Ambion (Austin, TX) can be used to detect DNase contamination in a sample. This kit detects DNase in a sample through the use of modified oligonucleotides that fluoresce upon cleavage by nucleases that may be within the sample. If nuclease activity is detected, the DNA sample can be exposed to high concentrations of guanidine hydrochloride (6–8 M) for 30 min or be subjected to an additional phenol–chloroform extraction for more thorough deproteinization of the sample. An ethanol precipitation should follow the additional deproteinization step to remove the phenol, chloroform, or chaotropic salts.

Fragmentation

Once purified and established as free of contaminating nucleases, genomic DNA must be fragmented to a desired size and made compatible for ligation into a prepared vector. Genomic DNA may be fragmented using enzymatic or mechanical means. Enzymatic fragmentation is accomplished using either restriction endonucleases or DNase I in the presence of manganese ions. Digestion with DNase I offers the ability to generate a more random pool of DNA segments compared to digestion with restriction endonucleases, which are biased based on the sequence of the genomic DNA and the recognition sites of the enzymes. Despite this, DNA digestion with restriction endonucleases is often preferred due to simplicity in reaction set-up and controllability. Appropriate restriction endonucleases for genomic DNA digestions are chosen based on five factors:

1. Frequency of cutting.
2. Buffer compatibility.
3. Ability to be denatured.
4. Methylation sensitivity.
5. The type of overhang that is produced.

Restriction endonucleases have known recognition sites ranging from 4 to greater than 30 nucleotide bp. Their frequency of cutting within a genome may be predicted if information is known about the genome sequence and the recognition sequence of the enzyme. Enzymes that cut with a high frequency in the genome, typically containing smaller recognition sequences, can be used to generate suitably random fragments by using partial digestions (**Figure 1**), or digestions that have not gone to completion. More than one restriction enzyme may be used for the partial digestion of DNA to ameliorate bias based on recognition sequence. Ideally, all of the restriction enzymes used in a digestion should have similar activity within a common reaction buffer and ability to be denatured to minimize DNA purification steps. Additionally, the restriction enzymes selected may be desired to be insensitive to dam methylation (methylation of the N6 position of the adenine in the sequence GATC) or dcm methylation (methylation of the cytosine at its C5 found in the sequences CCTGG and CCAGG), which may render the DNA resistant to cleavage. Lastly, restriction enzymes may be chosen based on the type of overhang that is left after cleavage. It can be arranged to have blunt ends or overhangs (called cohesive ends) that would be compatible with the prepared vector. This will eliminate a step to modify the ends of the DNA fragments prior to ligation.

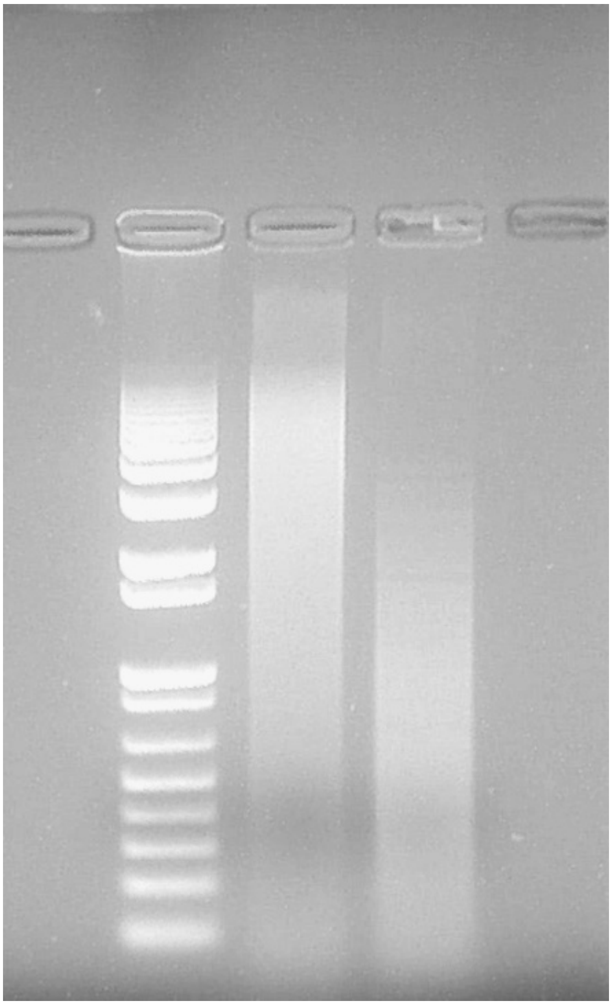

Figure 1 Restriction enzyme digestion of genomic DNA for 5 min (middle) and 16 h (right) next to DNA standard ladder (left).

Mechanical methods for DNA fragmenting offer the advantage of being unbiased toward DNA sequence and thus are useful for creating a more random pool of fragments. The main disadvantage associated with mechanically fragmenting DNA is the limitation on the size of fragments that can be generated as well as the extensive treatment that is required to repair the ends of the DNA necessary before they can be cloned into backbone vector. A French press, sonicator, clinical nebulizer, small gauge syringe, and HydroShear (Genomic Solutions Inc., Ann Arbor, MI) are common tools used to fragment DNA. Of these tools, the HydroShear was deliberately designed to shear DNA using hydrodynamic force and can be used to reproducibly create random fragments of DNA within a limited size range, independent of DNA concentration, starting length of DNA, and sample volume. Mechanical fragmentation of DNA will result in DNA that must be end-repaired to fill in or remove overhangs and restore 5′ phosphate groups. T4 DNA polymerase, Klenow fragment, or kits available to repair DNA ends may be used for this purpose. T4 polynucleotide kinase, when supplied with ATP, can be used to restore 5′ phosphate groups necessary for ligase activity.

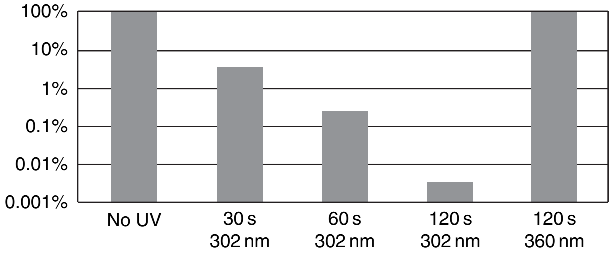

Figure 2 Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure (‘No UV’) or exposure to 302 nm UV light for 30, 60, or 120 s (‘30 s 302 nm, 60 s 302 nm, 120 s 302 nm’) or to 360 nm UV light for 120 s (‘120 s 360 nm’). Cloning efficiencies were calculated relative to nonirradiated pUC19 DNA. Reprinted with permission from Lucigen Corporation (www.lucigen.com).

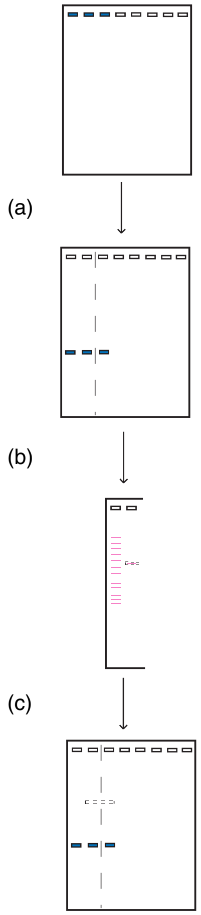

Figure 3 Digested DNA to be used in subsequent steps should not be exposed to UV light. After loading ladder in lane 1, a fraction of the digested DNA should be placed in lane 2 with the rest in lane 3. After running the gel (a), lanes 1 and 2 should be separated from lane 3 with a scalpel (b) and examined under UV light. The desired DNA fragment should be excised from lane 2, and both gel pieces should be brought together (c) to obtain the desired DNA fragment piece.

Agarose Gel Electrophoresis Used in Genomic Library Construction

Agarose gel electrophoresis is used to separate DNA by size or topology using an electric field that induces negatively charged DNA molecules to migrate to the positive pole through a porous matrix of agarose. In library construction, it is used in both vector preparation and isolation of genomic DNA pieces of a particular size. Circular vector migrates more quickly through an agarose gel than linearized vector with the same molecular weight and thus can be separated from vector that was linearized after digestion with a restriction enzyme. Vector that has ligated can also be separated from vector that has not been ligated and remained linear using gel electrophoresis. After genomic DNA has been fragmented, pools of generated segments are separated according to their molecular weight. DNA segments of less than 20 kbp can be sufficiently separated with a standard 1% agarose gel. The resolution of smaller pieces of DNA (<1 kbp) can be enhanced by increasing the percentage of agarose in the gel to 2%, while lowering the agarose content to 0.8% can enhance the resolution of larger pieces of DNA. If larger pieces of DNA need to be resolved, pulse field gel electrophoresis (which uses an alternating electric field) may be employed to achieve higher resolution.

Larger segments or fragile pieces of DNA that are sensitive to shearing should be resolved using gels made from low melting point agarose, which does not require vortexing or centrifugation steps to extract embedded DNA, but rather relies upon melting the gel at low temperatures followed by an incubation with β-agarase I to digest the agarose away from embedded DNA.

DNA within an agarose gel must be bound to a dye in order for visualization. The most commonly used dye to stain agarose gels is ethidium bromide (EtBr). EtBr is a fluorescent dye that intercalates between stacked bases of DNA. EtBr experiences excitation by UV radiation and emits energy at 590 nm, fluorescing with a red-orange color. Dye bound to DNA displays an almost 20-fold increase in fluorescent yield, thus allowing for the detection of as little as 10 ng of DNA. If EtBr is used to visualize DNA loaded onto an agarose gel, it is very important to minimize the amount of short wavelength UV light exposed to the DNA (**Figures 2** and **3**). Exposure to short wavelength UV light has been shown to damage DNA and decrease its cloning efficiency. In addition, EtBr is a potent mutagen and moderately toxic, so safer alternatives or dyes that can be seen without the aid of UV light are often desired. Alternative dyes include crystal violet, methylene blue, SYBR Safe(Invitrogen, Carlsbad, CA), or Nile blue.

Quantifying DNA and Determining Quality

It is important to be able to determine the amount and purity of DNA within a sample. DNA should be quantified following purifications steps to be sure of a sufficient yield prior to any further manipulations. The quality of DNA should also be monitored before initiating cloning steps to ensure that there are minimal contaminants that would interfere with the efficiency of cloning reactions.

The purity of a DNA sample can be accessed by calculating the ratio of absorbance at 260 nm to the absorbance at 280 nm measured by a spectrophotometer. Nucleic acids have a higher absorbance at 260 nm than at 280 nm. The reverse is true for proteins, which display higher absorbance at 280 nm than at 260 nm. The absorbance at each individual wavelength is thus influenced by the presence of both proteins and nucleic acids. Based on the extinction coefficients for both of these macromolecules, pure samples of DNA would have a A_{260}/A_{280} ratio of close to 2.0 and pure protein samples would have a A_{260}/A_{280} ratio of close to 0.6. Typically, a DNA sample with a A_{260}/A_{280} ratio of greater than 1.7 is acceptable for molecular cloning reactions.

The quantity of DNA can be measured via UV spectroscopy, fluorometry, or by comparison to a standard mass ladder on an agarose gel. Due to the simplicity, the concentration of DNA within a sample is frequently approximated based on the absorbance reading at 260 nm. The concentration is found through application of the Beer–Lambert law, which relates absorbance with concentration through the relationship

$$A = \varepsilon b C$$

where A = absorbance, b = pathlength of the sample cuvette, in units of length, ε = absorption coefficient in units of volume/mass/length, and C = concentration in units of mass per volume.

Using a standard spectrophotometer with a path length of 1 cm, an absorbance reading at 260 nm (A_{260}) of 1 equates to a concentration of approximately $1\,\mathrm{ng}\,\mu\mathrm{l}^{-1}$. As mentioned above, other molecules besides DNA that absorb at this wavelength, including proteins, RNA, and salts within the sample, can influence absorbance at 260 nm. Due to this phenomenon, the amount of DNA within a sample is usually confirmed or measured with a different method. Fluorometric measurements of DNA are more accurate than those obtained from UV spectroscopy and can detect smaller quantities of DNA. To quantify DNA, DNA-specific fluorescent stains, such as PicoGreen or SYBR Green I, are added to a DNA sample and the fluorescence of the sample is compared to the fluorescence of standards of known concentrations. Accurate DNA quantification can also be achieved by running an aliquot of sample along with a standard DNA mass ladder on an agarose gel and comparing DNA band intensities. This method is effective when quantifying distinctly sized pieces of DNA.

Ligation Reactions

A ligation reaction is required to bind fragmented genomic DNA into linearized vector. The most commonly used ligase, T4 ligase, is derived from the T4 bacteriophage and requires ATP as a cofactor and an available DNA $5'$ phosphate group on at least one of the two ligating DNA fragments. When setting up a ligation reaction, the moles of insert to moles of vector ratio may be varied to find optimal conditions. Lower ratios may result in inefficient ligation reactions while higher ratios increase the risk of ligating more than one insert per vector. Typically, insert to vector ratios are varied from 1:1 to 5:1. Blunt-ended ligations may perform best with higher ratios. A control ligation containing vector without insert should also be conducted to give an estimate of background clones that contain self-ligated vector. Ligases may or may not be required to be inactivated or purified from a reaction prior to transformation. Following the recommendations of the supplier of the ligase generally will give the best ligation and transformation results.

Transformation of Library DNA into Bacterial Host Strains

Naked DNA in solution can be transferred into a bacterial host strain via transformation of competent cells. Bacterial transformations with plasmid DNA is accomplished through heat shock of chemically competent cells or electroporation of electrocompetent cells. Transformation of chemically competent cells usually achieve 10^5–10^9 colony forming units (CFU) per μg of supercoiled DNA while electroporation of electrocompetent cells can yield up to $10^{10}\,\mathrm{CFU}\,\mu\mathrm{g}^{-1}$ of DNA.

Generally, the preparation of chemically competent cultures of *E. coli* involves treating exponentially growing cells with a salt solution, such as 0.1 M $CaCl_2$. Plasmid DNA is mixed with the cells and the plasmid DNA and cell suspension are heat shocked at 42 °C for a brief period, during which the cells can uptake the DNA.

While the exact mechanism of DNA uptake by this method is not fully known, it is believed that the swelling of the cells following treatment with the salt solution and the activation of heat shock genes are important in cells taking up DNA from their environment. Factors that influence the frequency of transformation include the purity of the reagents and water used, the viable cell density of the culture, and the trace contaminants that are found on glassware. Additionally, the number of times a culture has been passaged influences transformation efficiencies. Best results are obtained from cultures started directly from cryogenic freezer stock as opposed to cells that have been continuously passaged.

Electrocompetent cells are prepared by repeated washing of cells in low conductivity solutions such as 10% glycerol or $300\,\text{mmol}\,\text{l}^{-1}$ sucrose to reduce the ionic strength of the cell suspension. Electroporation works by using a transmembrane electric field pulse to create small holes, referred to as electropores, within the bacterial membrane through which DNA can pass. Electroporation conditions, such as pulse amplitude and duration, must be sufficient enough to generate electropores but not increased to the point at which the number and size of electropores detrimentally affect transformation efficiency by causing cell damage or death. The number of pulses, along with the pulse duration and amplitude, can be varied to empirically optimize conditions for various cell lines.

While many bacterial strains can be made competent, the protocols for preparing and manipulating competent *E. coli* are the most thoroughly worked out. Furthermore, competent *E. coli* can be obtained from commercial sources. Commercially available competent cells tend to yield transformation efficiencies several orders of magnitude greater than those typically achieved by standard laboratory preparations. Additionally, ligated DNA tends to have lower transformation efficiencies than supercoiled DNA, most likely due to DNA topology. For a combination of these reasons, the initial transformation step of transferring cloned library DNA to a host strain is usually conducted with *E. coli*, provided that the cloning vector used has an origin of replication that can be recognized by *E. coli* DNA polymerases. If desired, after this initial transformation step, the extracted supercoiled plasmid DNA can be prepared from transformed *E. coli* and then transformed into a different desired host cell line (**Figure 4**).

Increasing the Number of Transformants

When a large number of recombinant clones is required, the ligation reaction can be precipitated in the presence of yeast tRNA. Precipitation of ligation reactions prior to electroporation has been shown to give up to a 400-fold increase on the number of transformants. It is believed that the yeast tRNA alters or stabilizes the topology of the ligated DNA, increasing its efficiency of transformation. In this method, a 5 μl ligation reaction is mixed with 1 μg of yeast tRNA from a $1\,\mu\text{g}\,\mu\text{l}^{-1}$ solution, brought up to a total volume of 20 μl with ultrapure water, and then precipitated with twice the volume of cold absolute ethanol. The DNA is pelleted by centrifugation, washed twice with cold 70% ethanol, and allowed to air dry prior to resuspension in 1 μl of ultrapure water. This sample can then be transformed into competent cells.

Determining the Number of Transformants Needed for Coverage of an Entire Genome

The extent to which a library represents all sections of the genome can be statistically determined. The number of necessary transformants to have sufficient coverage or

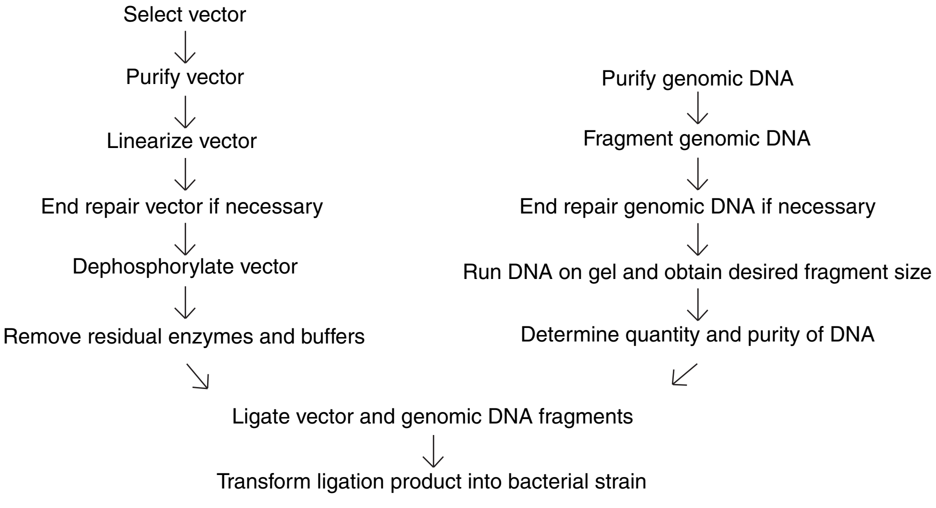
Select vector
Purify vector
Linearize vector
End repair vector if necessary
Dephosphorylate vector
Remove residual enzymes and buffers
Purify genomic DNA
Fragment genomic DNA
End repair genomic DNA if necessary
Run DNA on gel and obtain desired fragment size
Determine quantity and purity of DNA
Ligate vector and genomic DNA fragments
Transform ligation product into bacterial strain

Figure 4 Summary of steps necessary for constructing representative genomic libraries.

high probability of containing any given section of the genome is dependent upon the genome size and the size of genomic DNA inserts contained within a library. The Clarke–Carbon equation, based on the assumption that recombinant clones are distributed according to a Poisson distribution across the genome, can be used to determine the number of transformants needed to have a high probability of any given unique DNA sequence that would be present in a genomic library. The Clarke–Carbon equation can be written as

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where $N =$ number of recombinant clones required, $P =$ probability of finding a given unique DNA section, and $f =$ fraction of the total genome size that is contained within a single insert of the genomic library, equal to the size of the insert in bp per size of the genome in bp.

For *E. coli*, K12 genome with a 4 639 221 bp sequence, a genomic library containing 12 000 bp inserts would need 2667 transformants to have a probability of 99.9% of the library containing any given DNA sequence.

While the Clarke–Carbon equation is the most commonly used formula for determining the number of transformants, other equations, such as the Poisson distribution-based Lander–Waterman equation may also be used. The number of recombinant clones required may also be influenced by the application of the genomic library. Some applications requiring high amounts of overlap between DNA segments require higher numbers of recombinant clones while applications that require only sections of genes to be present, such as some hybridization studies, may require less transformants.

Current Strategies to Enhance Genomic Library Production

Large-scale sequencing projects, including the sequencing of entire genomes, require the construction of high quality and highly representational genomic libraries. These libraries should have minimal sequence bias and often it is desired to have clones with larger sized inserts to decrease the number of clones required to be sequenced. In order for genomic sequencing projects to be complete, all DNA of the organism must be included within the genomic library, including difficult to clone DNA that contain secondary structures, AT- or GC-rich regions, or DNA encoding strong promoters or toxic gene products. Additionally, it is desired for all insert DNA to be stable within cloning vectors so that the vector can be amplified or be available for other molecular biology experiments. To this end, a number of advances have been developed, particularly in the area of cloning vectors to improve cloning of genomic DNA fragments.

Linear vectors based on the coliphage N15, available commercially from Lucigen (Middleton, WI) such as pJAZZ vectors, have been shown to be stable for larges DNA segments (up to 30 kb) or DNA with difficult to clone secondary structure. The stability of these vectors is believed to be accredited to their lack of supercoiling and differences in replication compared to standard cloning vectors. Low copy number vectors, such as the pSMART or broad host range pRANGER-BTB series of vectors (available from Lucigen), have features that block transcription into and out of the multiple cloning site by the presence of transcriptional terminators and the lack of constitutive promoters. These vectors have been shown to be several times more stable for cloning random DNA fragments than pUC vectors thus minimizing cloning gaps caused by difficult-to-clone DNA. Another recently developed vector intended to facilitate sequencing, pLEXX-AK (also available from Lucigen), is designed to clone two inserts per vector, thus reducing the downstream labor involved in processing clones prior to sequencing.

Additional advances in constructing genomic libraries come from a reduction in the amount of work required. Many molecular biology suppliers now offer kits to aid in genomic library construction. These kits typically contain pre-processed vector that has already been linearized and dephosphorylated, along with prepared competent cells, reducing the amount of user time required to create a genomic library. Commercially prepared vectors typically promise much lower background empty vector than is usually obtained when cloning vectors are prepared locally.

Sample Protocol to Construct a 4 kb *Pseudomonas aeruginosa* PAO1 Library with Vector PBTB-1 Within an *E. coli* Host Strain

Supplies and Reagents Needed

All kits and products should be used according to the recommendations of the suppliers unless otherwise noted.

- Incubator set at 37 °C.
- Water bath set at 37 °C.
- Heat block.
- Milliliter conical tubes.
- Yeast tRNA (1 mg ml^{-1} in ultrapure water) (Sigma-Aldrich, St. Louis, MO).
- Ultrapure water (Invitrogen).
- 100% ethanol.

- 70% ethanol.
- *E. cloni* 10G (F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *ϕ*80*dlac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1*ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K *rps*L *nup*G λ-*tonA*) (Lucigen) containing plasmid pBTB-1 (*β*-lactamase resistance, low copy number).
- *P. aeruginosa* PAO1.
- Luria–Bertani (LB) broth.
- YT agar plates supplemented with 100 μg ml^{-1} carbenicillin.
- Sterile-filtered stock solution of carbenicillin (100 mg ml^{-1}).
- HiSpeed Plasmid Midi kit (Qiagen, Valencia, CA).
- 500/G Genomic-tips (Qiagen).
- Genomic DNA Buffer Set (Qiagen).
- MinElute Gel Extraction Kit (Qiagen).
- QIAprep Spin Miniprep Kit (Qiagen).
- Nuclease-free water (Ambion).
- Agarose gel electrophoresis apparatus.
- 1 × TAE buffer (50×: 242 g Tris base 57.1 ml acetic acid 100 ml 0.5 M EDTA. Add deionized water to 1 l and adjust pH to 8.5) to be used for all electrophoresis steps and in preparing agarose gels.
- Molecular biology grade agarose (Sigma-Aldrich).
- Low-melting point agarose (Promega, Madison, WI).
- 10 mg ml^{-1} stock solution of EtBr.
- 10% sodium dodecyl sulfate solution (Ambion).
- GELase Agarose Gel-Digesting Preparation (Epicentre Biotechnologies, Madison, WI).
- *Hinc*II (New England BioLabs, Ipswich, MA).
- *Rsa*I (New England BioLabs).
- *Hae*III (New England BioLabs).
- Antarctic Phosphatase (New England BioLabs).
- T4 DNA ligase (Lucigen).
- High DNA Mass Ladder (Invitrogen).
- 1 kb Plus DNA Ladder (Invitrogen).
- UltraClone DNA Ligation & Transformation Kit containing ELITE DUOS (Lucigen) (contains ligase and *E. cloni* ELITE 10G electrocompetent cells).

Vector Preparation

1. Inoculate 100 ml of LB broth supplemented with 100 mg l^{-1} carbenicillin and allow to incubate overnight at 37 °C, 225 rpm.
2. Prepare plasmid from overnight culture with a Qiagen HiSpeed Plasmid Midi kit, eluting with nuclease-free water.
3. Quantify vector by visual comparison to a High DNA Mass Ladder run on a 1% agarose gel stained with 0.5 μg ml^{-1} ethidium bromide. If vector is too dilute (>100 μg ml^{-1}), concentrate the sample with a speedvac.
4. Blunt cut 1 μg of vector with 10 units of *Hinc*II. Heat inactivate the reaction.
5. Allow the sample to equilibrate to 37 °C.
6. Add Antarctic Phosphatase reaction buffer to a final concentration of 1 × and 5 units of Antarctic Phosphatase. Following dephosphorylation, heat inactivate the sample.
7. Allow the sample to cool to room temperature.
8. Add 10 × T4 DNA ligase Buffer containing ATP to a final concentration of 1 × and 2 units of ligase. Allow the reaction to proceed for 2 h at room temperature prior to heat inactivation.
9. Separate circular and linearized vector on a 1% agarose gel stained with 0.5 μg ml^{-1}, running the sample adjacent to a 1 kb Plus DNA ladder.
10. Extract the linearized band from the gel with a clean razor blade, using care to minimize UV exposure of the DNA.
11. Purify vector DNA from the agarose gel using a MinElute Gel Extraction Kit, eluting with nuclease-free water.
12. Quantify the vector DNA by visual comparison to a High DNA Mass Ladder run on a 1% agarose gel stained with 0.5 μg ml^{-1}.
13. Vector may be stored frozen at −80 °C.

P. aeruginosa PAO1 Genomic DNA 4 kb Insert Preparation

1. Inoculate 100 ml of LB media with *P. aeruginosa* PAO1 and allow to incubate overnight at 37 °C, with shaking at 225 rpm.
2. Extract genomic DNA using 500/G Genomic-tips and Genomic DNA Buffer Set.
3. Quantify the genomic DNA by visual comparison to a High DNA Mass Ladder run on a 1% agarose gel stained with 0.5 μg ml^{-1} EtBr.
4. Partially digest 5 μg of DNA with 10 units each of blunt-cutting *Rsa*I and *Hae*III for 1 h.
5. Add 10% SDS to the reaction to a final concentration of 1×.
6. Resolve DNA fragments on a 1% low melting point agarose gel stained with 0.5 μg ml^{-1} EtBr, running the sample adjacent to a 1 kb Plus DNA ladder.
7. Excise with a clean razor blade the section of the gel containing the 4 kb fragments of DNA, using caution to minimize UV exposure of the DNA.
8. Recover DNA using a GELase Agarose Gel-Digesting Preparation.
9. Quantify and verify length of the genomic DNA fragments by visual comparison to a High DNA Mass Ladder run on a 1% agarose gel stained with 0.5 μg ml^{-1} EtBr.

Ligation and Transformation

1. Following the directions included in the UltraClone DNA Ligation & Transformation Kit, ligate insert DNA to vector with a 3:1 insert to vector ratio.
2. Heat inactivate the reaction.
3. Precipitate the ligation by adding to the reaction 2 µl yeast tRNA (1 mg ml^{-1}), 28 µl ultrapure water, and 100 µl 100% ethanol.
4. Vortex the mixture and allow it to cool at −20 °C for 15 min.
5. Pellet by centrifuging for 15 min (13 000 rpm and 4 °C).
6. Carefully remove the supernatant.
7. Wash the pellet with 70% ethanol.
8. Centrifuge the sample again (13 000 rpm and 4 °C).
9. Remove the supernatant and allow the pellet to air dry.
10. Resuspend the pellet in 2 µl of ultrapure water.
11. Transform the precipitated ligation reaction into electrocompetent *E. cloni* cells included in the kit following the suggestions of the supplier.
12. Allow cells to recover for an hour at 37 °C with shaking at 225 rpm.
13. Make a 1/100 dilution of the transformed cells prior to plating.
14. Plate transformed cells and dilution onto YT agar plates containing 100 µg ml^{-1} carbenicillin.
15. Incubate the plates overnight at 37 °C.
16. Count the number of colonies on the plates onto which the dilution was plated. Calculate the number of transformants based on this count.

Calculating the Number of Recombinant Clones Needed for a 4000 bp Library

1. For a representational library (99.9% probability of containing any given DNA sequence), calculate the number of transformants needed using the Clarke–Carbon equation (see 'Determining the number of transformants needed for coverage of an entire genome'). The size of the *P. aeruginosa* PAO1 genome is 6 264 404 bp.

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

with $P = 0.999$ and $f = 4000/6\,264\,404$, the number of transformants needed $N = 10\,815$.

Confirmations

1. Fill ten sterile 15 ml conical tubes with 5 ml of LB supplemented with 100 µg ml^{-1} carbenicillin.
2. Pick ten colonies chosen at random from the plated library transformation to inoculate the ten conical tubes.
3. Allow the cultures to incubate overnight at 37 °C and 225 rpm.
4. Prepare the vectors from these cultures using a QIAprep Spin Miniprep Kit.
5. Vector DNA may be run on a 1% agarose gel to check for appropriately sized insert or be sequenced with primers designed to extend into the multiple cloning site.

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http://www.lucigen.com – Lucigen Corporation
http://www.qiagen.com – Qiagen

Gram-Negative Cocci, Pathogenic

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Defining Statement
Infection by *Neisseria meningitidis* and *Neisseria gonorrhoeae*
Molecular Mechanisms of Infection
Antigens
Natural Immunity
Prevention
Conclusion
Further Reading

Glossary

capsules An external layer usually consisting of a complex polysaccharide coating the surface of many species of pathogenic bacteria.

DNA-mediated transformation Pathogenic *Neisseria* are able to specifically transport DNA from the environment into the cell and by homologous recombination integrate it into the genome.

meningitis Inflammation of the meninges that are membranes covering the brain and the spinal cord, resulting from a bacterial or viral infection.

meningococcemia Infection of the bloodstream by meningococci in the absence of meningitis.

petechial skin lesions Small purplish spots on the skin caused by a minute hemorrhage.

porins Protein molecules found in outer membranes of Gram-negative bacteria serving as channels for the diffusion of water and small molecular weight solutes.

Abbreviations

CDC	Center for Disease Control
CECAM	CEA-related cell adhesion molecule
ET	electrophoretic types
fMLP	formyl methionyl-leucyl-proline
GlcNAc	N-acetyl glucosamine
Hep	heptose
HSPG	heparan sulfate proteoglycan
IgV	immunoglobulin variable
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
KDO	keto-deoxyoctulosonic acid
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
NANA	N-acetyl-neuraminic acid
N-CAM	neural cell adhesion molecule
PE	phosphoethanolamine
PID	pelvic inflammatory disease
PMN	polymorphonuclear
Rmp	reduction modifiable protein
VDAC	voltage-dependent anionic channel

Defining Statement

Gram-negative cocci are almost invariably isolated from nasopharyngeal cultures of human beings. The majority of these are commensal species. Members of the genus *Neisseria* are nonmotile and oxidase- and catalase-positive Gram-negative diplococci that utilize sugars oxidatively. Members of this genus are differentiated primarily on the basis of colony morphology and their ability to utilize various carbohydrates. *Neisseria gonorrhoeae* can utilize only glucose, whereas *Neisseria meningitidis* utilizes glucose and maltose. *Neisseria lactamica* resembles *N. meningitidis* but can also utilize lactose. On rare occasions, bacteria belonging to a few of these species are able to invade the human host and cause disease; this is summarized in **Table 1**.

Infection by *Neisseria meningitidis* and *Neisseria gonorrhoeae*

Local Infection

Among the *Neisseria* and related organisms listed, *N. meningitidis* and *N. gonorrhoeae* are primary pathogens, namely, organisms that are able to cause disease in an

Table 1 Commensal and pathogenic Gram-negative cocci

Species	*Distribution*	*Pathogenicity*
Commensal species		
Neisseria lactamica	Very commonly colonizes nasopharynx of young children	Very rare instances of sepsis and meningitis
Neisseria subflava family: flava, sicca, perflava	Commonly found in nasopharyngeal cultures	
Neisseria cinerea	Nasopharynx	Very rarely peritonitis in dialysis patients
Moraxella catarrhalis	50% of children are carriers during the winter months	Third most common cause of acute otitis media and sinusitis in young children. Can cause acute exacerbations of chronic bronchitis in adults. Sepsis very rare
Moraxella lacunata	Nasopharynx	Formerly common cause of conjunctivitis and keratitis; now rare
Moraxella bovis	Human and bovine nasopharynx	Causes outbreaks of bovine keratoconjunctivitis
Moraxella nonliqefaciens	Nasopharynx	
Moraxella osloensis	Nasopharynx	Occasionally causes invasive disease
Moraxella canis	Canine and feline oral cavity	Infections following dog and cat bites
Kingella kingi	Commonly in nasopharyngeal cultures	Occasionally causes arthritis, osteomyelitis and sepsis in children below age 2
Primary pathogens		
Neisseria meningitidis	Nasopharynx, rarely genitourinary tract	Sepsis and meningitis
Neisseria gonorrhoeae	Genitourinary tract, less commonly rectum and pharynx	Gonorrhea, sepsis, septic arthritis and dermatitis, rarely meningitis

otherwise healthy host. DNA hybridization and genomic sequences of *N. meningitidis* and *N. gonorrhoeae* indicate that they are extremely closely related organisms. It is therefore not surprising that the pathogenetic strategies of the organisms are for the most part very similar. Both are able to cause infection of mucous membranes. *N. meningitidis*, transmitted by droplets from the respiratory tract of an infected individual, usually infects the mucous membranes of the nasopharynx, but has been isolated on occasion from genitourinary sites. The nasopharyngeal infection with *N. meningitidis* is most often asymptomatic and is self-limited, with the infection lasting for a few weeks to months. This colonization is referred to as the carrier state and is quite common. During the winter months, the frequency of carriers is usually 10% or greater. In populations that live in close contact, such as in boarding schools and military recruit camps, the carrier rate not infrequently will exceed 50%.

Gonococci are transmitted most often by sexual contact, infect the genitourinary tract, but are also quite frequently isolated from rectal and pharyngeal cultures. In 2005, 340 000 cases were reported by the Center for Disease Control (CDC). The infections of the genitourinary tract are most often symptomatic, whereas oropharyngeal infections generally cause no symptoms. The conjunctivae are susceptible to gonococcal infection, particularly in neonates who acquire it when passing through an infected birth canal. Historically, this infection, ophthalmia neonatorum, was extremely common and was the major cause of acquired blindness until the general acceptance of the preventive Credè procedure, first introduced in 1881 and consisting of the instillation of drops of a 1% silver nitrate solution in the eyes of newborns. This has been replaced by the use of less irritating antibiotic ointments.

Extension of Local Infection

Genitourinary gonococcal infection in women usually involves the endocervix and, with lesser frequency, the urethra, the rectum, and the pharynx. As many as 50% of the infections may be asymptomatic or with insufficient symptoms to prompt the person to seek medical attention. In at least 10% of infections, there is early extension to the uterine cavity (endometritis), and subsequently ascending infection of the fallopian tubes (pelvic inflammatory disease (PID)). PID is the major complication of gonorrhea. Most often it requires hospitalization for differential diagnosis and treatment and causes tubal scarring, resulting in infertility and tubal pregnancies. The latter is an obstetric emergency always causing death of the fetus and of about 10% of the mothers.

Infections in men, while sometimes asymptomatic, most often cause sufficient symptoms to drive the affected individual to seek medical attention. In the days prior to the availability of antibiotic therapy, epidydimitis occurred in about 10% of cases, but this complication as well as prostatitis are rarely seen today. However, gonorrhea, as well as other sexually transmitted diseases, increases severalfold the probability of transmitting or acquiring HIV infection.

Bacteremic Infection

Both *N. meningitidis* and *N. gonorrhoeae* first colonize the epithelial surface and are able to traverse epithelial cells by mechanisms described below. Once the organisms are in the subepithelial space, both the meningococcus and the gonococcus may invade the bloodstream. With *N. meningitidis*, bloodstream invasion and subsequent invasion of the meninges of the brain result in meningitis, which is a very dangerous bacterial infection and can very rapidly lead to death even with optimal antibiotic and supportive therapy and is usually accompanied by disseminated intravascular coagulation, a petechial rash and high levels of circulating endotoxic lipopolysaccharide (LPS) and capsular antigen. In the United States, there are about 1500 reported cases of meningococcal meningitis each year, and now that meningitis due to *Haemophilus influenzae* type b is a vanishing disease as a result of widespread acceptance of vaccination, meningococcus is the leading cause of meningitis.

Meningococcal disease occurs worldwide as an endemic disease principally in infants 3 months or older and in young children with a rate of 2–5 cases per 100 000 population. The incidence is seasonal, with winter and spring having most cases. However, meningococcal disease can also occur in epidemic form where the rate of disease can rise as high as 200–1000 cases per 100 000. During epidemics, the peak incidence shifts to an older age group, children aged 5–7 years. During the first half of the twentieth century, epidemics caused by group A meningococci occurred in the United States about every 12 years. Since World War II, there has not been a major epidemic in the United States, but they have occurred in many other parts of the world, notably Brazil, China, Finland, Russia, Mongolia, and New Zealand. However, the area of the world most severely affected is Africa in the so-called meningitis belt that extends through all of the sub-Saharan countries from the Sahel to the rain forest region. In this region, major epidemics affecting tens of thousands of inhabitants have occurred every 3–4 years. In the winter season of 1995–1996, there were 250 000 cases in West Africa. In the year 2007, there were about 27 000 cases in that area.

In the case of the gonococcus, it is estimated that 1–2% of patients with gonorrhea do have invasion of the bloodstream, which can in rare instances be as fulminant as meningococcal infection, but usually has a much more benign course. The disease most often presents with fever, arthritis, and petechial, hemorraghic, pustular, or necrotic skin lesions. The disease can also present as monoarticular arthritis unaccompanied by rash. If untreated this can progress to septic arthritis that may cause severe damage to the affected joint.

Treatment

Meningococcal infection is a medical emergency, and the earlier the effective antibiotic treatment is initiated the better the prognosis. The mortality rate for meningococcal meningitis is generally about 10%, but is much higher in meningococcemia and shock. The drug of choice for treatment remains intravenously administered penicillin G in very high doses. However, since meningitis due to pneumococcal and *H. influenzae* infection can present with a very similar clinical picture and because these organisms not infrequently are resistant to penicillin, third-generation cephalosporins together with vancomycin are recommended as initial therapy in children until the meningococcal etiology has been established. Patients need to be hospitalized because the course of the disease is unpredictable and supportive therapy is frequently needed.

The treatment of gonococcal infection has changed over the past decades due to the development of partial or complete resistance of this organism to many antibiotics. Recently, gonococcal strains resistant to fluoroquinolones have become quite common, and the CDC counsels against their use and recommends third-generation cephalosporins. In addition, coinfection with *Chlamydia trachomatis* is a very common occurrence, and the treatment should also eliminate this organism.

Molecular Mechanisms of Infection

One of the problems in the study of neisserial disease is that these organisms are restricted to human beings, and animal models have provided relatively little information on the pathological events occurring during the various stages of the infection. The human disease has been most closely simulated by organ culture systems, in particular by one employing fallopian tubes that are obtained from women undergoing medically indicated hysterectomies. The epithelium lining the fallopian tubes consists principally of two kinds of cells, mucus-secreting cells and cells that bear cilia and beat in unison to move the mucus layer. When gonococci are added to the explants *in vitro*, the first discernible interaction is that the gonococci attach by means of long hair-like projections known as pili to the surface of the mucus-secreting cells, but not to the ciliated cells. This is a distant attachment between the bacteria and the cell surface and occurs about 6 h following inoculation. Then over the next 12–18 h, this distant attachment converts to a very close attachment that may be due to pilus retraction (see below). The close attachment is believed to be favored by a set of outer membrane proteins called opacity (Opa) proteins (discussed below). These interactions initiate a signaling cascade that causes the epithelial cells to engulf the gonococci, transport the bacteria through the body of the cell in vacuoles, and egest them on the basal part of the cells onto the basement membrane. Later in the infection (between 24 and 72 h), toxic phenomena occur that result in expulsion of the ciliated cells from the epithelium. If meningococci are placed on human fallopian tubes, the same events occur but over a shorter period of time. However, these events are not seen with commensal *Neisseria* species, or if the fallopian tube is not of human origin. Cervical biopsies obtained from infected patients as part of diagnosis or treatment of cancer show a microscopic picture that is quite similar.

The events transpiring in the course of the model infection have been the focus of research in order to understand meningococcal and gonococcal disease in molecular terms. Obviously, the establishment of the mucosal infection depends on cross talk between the bacterium and the host cells, and this is discussed in 'Antigens'. It is noteworthy that compared to the other pathogens such as *Salmonella, Shigella*, and *Listeria*, where invasion occurs quite promptly, there is a long lag in the invasion by the *Neisseria* as if some slow inductive events need to occur in the host cell or the organism or both.

Antigens

The surface antigens of the *Neisseria* have been extensively studied to gain an understanding of the molecular steps underlying the pathogenesis of these diseases as well as to identify candidate molecules for inclusion in vaccines.

Pili

Most peripheral on the surface are pili that are hair-like appendages with a diameter of about 8 nm that emanate from the outer membrane of gonococci and are several bacterial diameters long. Pili have a number of functions that include adherence, motility, and participation in DNA-mediated transformation. Pili consist of the helical aggregation of a single kind of protein subunit of about 18 000 molecular weight (MW) known as pilin. Neisserial pili belong to the class of type IV pili found in many bacterial species, notably *Pseudomonas aeruginosa, Moraxella* sp., and *Vibrio cholerae.* The study of pili on gonococci is simplified by the fact that their expression imparts a distinctive appearance to the gonococcal colony as it grows on agar. Observation of this variability indicates the gonococcus has the ability to turn on and off pilus expressionat a very high frequency.

Gonococci freshly isolated from patients invariably are piliated. It is known from challenge studies of volunteers that only piliated strains are capable of causing infection. Stable nonreverting pilus-negative strains do not cause infections. Pili are antigenically highly variable. No two strains of gonococci appear to have the same pili, and pili expressed by a single strain of gonococcus maintained in the laboratory over time repeatedly change their antigenicity. The mechanism of antigenic variation has been elucidated. Generally, the gonococcus possesses a single genetic locus expressing the pilin, which is called *pilE.* This locus contains a complete pilin structural gene with its promoter. In addition to the expression locus, gonococci also have several silent loci that contain pilin genes that lack the promoter sequences and portions corresponding to the beginning of the protein coding frame. These incomplete genes are efficiently shuttled by homologous recombination into the *pilE* expression site, causing the production of a large number of serological variant pili by a single strain of gonococcus over a period of time. If the new antigenic variant pilin can be assembled into intact pili, then an antigenic variation step will occur; this occurs at a rate of 1 in a 1000 cells per cell division. If the new pilin cannot be assembled into an intact pilus, then the organism is pilus negative, but is able to revert to pilus positive as soon as a gene copy that can be assembled is recombined into the expression locus. Thus, the recombinational mechanism accounts both for on–off variation and for antigenic variation. This is obviously a remarkably complex genetic mechanism for varying this protein, and the only conceivable evolutionary pressure to force the development of this system is of course the human immune system.

The genes that are involved in the biogenesis of type IV pili of meningococci and gonococci have been characterized, and it is recognized that this is a dynamic process with extension being mediated by *pilF* and retraction by *pilT.* Extension appears to be favored by six additional genes *pilC* and *pilH–L.* Mutations of any of the genes in the *pilH–L* cluster prevent aggregation of the bacteria and also adhesion to epithelial cells. *pilT*-mediated retraction gives rise to substantial mechanical force, imparts so-called twitching motility to the bacteria, and may be important in converting distant attachment to epithelial cells to close attachment. PilC is a pilus-associated protein of about 110 000 MW, not only favoring the assembly of pili, but also imparts the ability to adhere to epithelial cells since it is present at the tip of the pili. The cellular receptors that are recognized by pili are still controversial, but one candidate proposed is the complement control protein CD46, which also serves as the receptor for measles virus.

Crystallographic and other structural studies have shown that pili consist of a helical aggregate of pilin subunits and that, remarkably, the exposed surface of the pilus cylinder consists of the variable domains, while the constant regions of the pilin molecule are buried within the cylinder. In addition, pilin subunits bear a trisaccharide consisting of two hexose units (galactose) and an unusual diamino sugar, which depending on the genetic background may be 2,4-diacetamido-2,4,6-trideoxyhexose or glyceramido acetamido trideoxyhexose linked to a particular serine in the pilin sequence. There is also a phosphoethanolamine (PE) linked to another serine residue. The biological role of these posttranslational modifications is not known.

Capsules

Meningococci are classified into serogroups on the basis of the chemical nature of the capsular polysaccharide they express (**Table 2**). Thirteen serogroups have been described, and epidemiologically, groups A, B, C, Y, and W-135 are the most important because they are the cause of nearly all cases of meningitis and meningococcemia. Group A is the classical epidemic type having been responsible for the recurrent epidemics in the United States in the first half of the twentieth century, in Finland and Brazil around 1970, and continuing to be the major epidemic type in the African meningitis belt. In 2002, there were a very considerable number of cases of meningitis due to serogroup W-135, in Burkina Faso, but more recently, group A has again predominated. Group B epidemics disease has been seen in Cuba, Norway, and most recently in New Zealand. Group C epidemic disease has been a problem recently in the United Kingdom, Spain, and Canada.

Table 2 Chemical structure of meningococcal capsular polysaccharides

Serogroup	*Repeating unit in capsular polysaccharide*
A	→ 6)-α-*N*-acetyl mannosamine-1-phosphate; *O*-acetyl C3
B	→ 8)-α-*N*-acetyl neuraminic acid-(2 →
C	→ 9)-α-*N*-acetyl neuraminic acid-(2 → ; *O*-acetyl C8
X	→ 4)-α-*N*-acetyl glucosamine-1-phosphate
Y	→ 6)-α-glucose-(1 → 4)-*N*-acetyl neuraminic acid-(2 → ; *O*-acetyl C7
Z	→ 3)-α-*N*-acetyl galactosamine (1 → 1)-glycerol-3-phosphate
29E	→ 3)-α-*N*-acetyl galactosamine (1 → 7)-β-KDO (2 → ; *O*-acetyl C4 or C5 of KDO
W-135	→ 6)-α-galactose-(1 → 4)-α-*N*-acetyl neuraminic acid-(2 → ; *O*-acetyl C7

KDO, keto-deoxyoctulosonic acid.

The presence of the capsular polysaccharides protects the organism from the natural defense of the host such as phagocytosis by white cells and killing mediated by complement via the alternative pathway. Meningococci isolated from systemic infection are encapsulated. However, the presence of antibodies to the capsular polysaccharides is protective, and these antigens are the basis of the meningococcal vaccines (discussed below). The locus for the biosynthesis of capsular polysaccharide has been characterized and is encompassed by about 25 kb of the genome. The right and left sides of the locus are conserved among serogroups and are concerned with common biosynthetic steps such as the addition of lipid carriers and the export of the product from the cytoplasm to the exterior. The middle portion of the locus differs between serogroups and contains the enzymes responsible for the biosynthesis of the activated sugar intermediates for the particular serogroup as well as the specific polymerase assembling the polysaccharide. The modular construction of the locus permits in the laboratory conversion from one serogroup to another by DNA-mediated transformation, and this phenomenon, on rare occasions, appears to have occurred in nature.

Outer Membrane Proteins

The outer membrane of the pathogenic *Neisseria* like that of other Gram-negative bacteria consists of a lipid bilayer with the outer leaflet consisting principally of LPS. The outer membrane contains a number of integral membrane proteins of which quantitatively the porins are predominant. The nomenclature of the neisserial outer membrane proteins has evolved with increasing knowledge of these proteins and is summarized in **Table 3** to assist the reader interested in the earlier literature.

Porins

The porins of the pathogenic *Neisseria* like those of *Escherichia coli* are postulated to consist principally of β-pleated sheets arranged perpendicularly to the membrane with loops exposed to the cytoplasm and eight loops exposed on the surface of the organism. Each functional

Table 3 Outer membrane proteins of pathogenic *Neisseria*

Meningococcal proteins	*Gonococcal proteins*	*Genetic designation*
Class 1	No homolog	*porA*
Class 2	Protein I, PI, PIB	*porB*
Class 3	Protein I, PI, PIA	
Class 4	PIII, Rmp	*Rmp*
Class 5	PII, opa	*opaA*–*opaJ*, or opa_{50}–opa_{60}
Opc	No functional homolog	*Opc*

porin consists of a trimer of the porin subunit. Neisserial porins not only serve as channels through which water and solutes of less than 1000 MW can diffuse through the outer membrane, but also play an active role in pathogenesis. The meningococcus contains two genetic loci that code for the production of outer membrane porins called *porA* and *porB*. The gonococcus lacks a *porA* locus, but possesses the *porB* locus that gives rise to porins that are very similar in amino acid sequence to the meningococcal proteins. The gonococcal porins vary antigenically primarily in the surface-exposed loops and fall into two main classes referred to as PIA and PIB. PIA strains predominate among gonococci isolated from the bloodstream and apparently have an increased capacity to invade the bloodstream and cause disseminated gonococcal disease. PIA strains also tend to be resistant to the bactericidal action of normal human serum. Strains that cause ascending infection of fallopian tubes are of the PIB type.

Biophysical studies in artificial lipid membranes indicate that the gonococcal porins are unusual among Gram-negative porins in that they are somewhat anion-selective and voltage-sensitive. Voltage sensitivity means that when the protein is in a membrane, the channel will be modulated by the potential across that membrane, such that at low membrane potentials, the porin molecules will be open, and as the voltage is raised, the probability that the porin molecule is closed increases. In addition, it has been shown that the porins are able to bind GTP and certain other phosphate compounds, and this binding also favors the closing of the porin channel. The neisserial porins readily transfer from the outer membrane of living gonococci to foreign membranes including human cells. PIA porins transfer more readily than PIB into artificial membranes. Gonococci with PIA porins (but not PIB) are readily ingested by Chang conjunctival cells and other human epithelial cell lines as long as the phosphate concentration in the assay medium is below 1 mM (the concentration prevailing in human serum and secretions). GTP is able to inhibit this PIA-mediated invasion at very low concentration (50% inhibition at 0.03 mM).

The earliest studies on the effect of porins was done with human polymorphonuclear (PMN) leukocytes. Within seconds after the addition of purified porin to PMN, the membrane potential of these cells becomes hyperpolarized due to the chloride ion movement. Shortly thereafter, the membrane potential returns to baseline, presumably because the porin channels adjust to this hyperpolarization by closing, and the active ion pumps of the cells reestablish the baseline potential. However, the cells are altered and this is evident when these cells are subsequently exposed to a stimulus such as formyl methionyl-leucyl-proline (fMLP). Normally, fMLP causes an immediate depolarization of the membrane. However, with porin channels present in the membrane, fMLP induces a prolonged hyperpolarization. Porin also markedly inhibits the aggregation of PMN. Degranulation in response to fMLP, leukotriene B4, or complement component C5a is also blocked, but is normal when induced with phorbol myristate acetate. However, there is no inhibition of superoxide generation in response to these signals.

The neisserial porins transferred into the cell membrane of HeLa cells are further transported to mitochondrial membranes where they associate with voltage-dependent anionic channel (VDAC). Such cells show increased resistance to undergo apoptosis, thereby preserving the intracellular niche for the proliferation of the neisseria as they transit through epithelial cells to reach the submucosa.

Rmp

All strains of gonococci produce an outer membrane protein originally designated PIII. This protein migrates on a SDS–PAGE with an apparent MW of 32 000 when exposed to reducing agents such as ß-mercapto ethanol, and with a MW of 31 000 when not reduced. Hence, the protein has been named reduction modifiable protein (Rmp). In contrast with other outer membrane proteins, Rmp is a highly preserved antigen showing little, if any, variation among strains. The sequence of Rmp has substantial homology with OmpA, a protein that is universally present in all enterobacterial species. Rmp is also present in meningococci where it was originally named class 4 protein and it is almost identical to the Rmp of gonococcus.

It has been found that complement-fixing immunoglobulin G (IgG) antibodies to Rmp are present in the sera of at least 15% of normal human beings with no history of prior gonococcal infection. These antibodies arise in response to the meningococcal carrier state and also by contact with the enterobacterial flora. Surprisingly, these antibodies do not mediate serum killing or opsonization of gonococci, but instead block the ability of normally bactericidal antibodies directed to other surface antigens to exert their function. Anti-Rmp antibody is a powerful blocking antibody, inhibiting the activity of other bactericidal monoclonal antibodies directed to a number of different surface proteins or LPS. In a prospective epidemiologic study of a population at very high risk of acquiring sexually transmitted diseases, it has been demonstrated that the presence of anti-Rmp antibodies significantly increases the risk of gonococcal infection, demonstrating the inhibitory role of blocking antibodies in the local mucosal infection. The blocking activity of anti-Rmp antibodies is not seen with meningococci, perhaps because this organism expresses quantitatively less Rmp. The molecular mechanism by which anti-Rmp antibodies act as blocking antibodies is not yet understood.

Opa proteins

Pathogenic *Neisseria* express another surface-exposed class of outer membrane proteins that are called opacity proteins (Opa) because their expression leads to distinctive changes in the translucency of the colonies on agar. In the gonococcus, there are about a dozen *opa* genes while in the meningococcus there are usually four. The expression of Opa protein can turn off and on at high frequency because all of the *opa* genes have a variable number of pentameric repeats of the sequence CTCTT between the ATG initiation codon and the remainder of the protein that is subject to rapid change due to slipped strand mispairing during replication. Thus, the expression of this class of proteins is controlled at the level of protein translation. Gonococci freshly isolated from the blood of patients with disseminated gonococcal infection do not express Opa. The same is true of isolates from PID. Strains from males with genitourinary disease usually express Opa protein. Most remarkably, in young women not on the pill, the gonococci that can be isolated from the cervix vary so that at the time of ovulation the isolates express Opa, while at the time of menses they do not. This mechanism of translational mechanism for variation has now been seen with other neisserial antigens and is also seen in a number of other mucosal pathogens, notably *H. influenzae* and *Helicobacter pylori.* Many different *opa* genes have been sequenced and have been distinguished either by naming them *opaA–opaJ* or by adding a numerical subscript (see **Table 3**). The mature proteins coded by these loci are all about 250 amino acids in length. The genes are highly homologous except for two regions that are highly variable and a smaller region that has lesser variation. Gonococi have about 12 *opa* loci while meningococci have 3 to 4. The differences between the proteins in the content of basic amino acids is noteworthy and the pI of the proteins range from about 7.0 to 10.0.

This class of proteins promotes adhesiveness of gonococci and meningococci to epithelial cells in tissue culture or to human PMN. The ligand specificity of the Opa proteins has been defined on a molecular level. The majority of Opa proteins react with several proteins that are members of the CECAM family (CEA-related cell adhesion molecule). CEA was originally described as a colon-cancer-associated antigen, and tests for blood levels of CEA antigen are used to clinically monitor the progression of colon cancer. There are about 20 related proteins known; their genes are clustered on human chromosome 19, they belong to the Ig superfamily, and they have an N-terminal domain homologous to Ig variable (IgV) domain and a variable number of domains with homology to Ig constant regions. Some are transmembrane proteins with cytoplasmic tails, while CEA (CECAM5) is GPI linked. The cytoplasmic tails of some members of the family contain a functional immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibition motif (ITIM). The proteins are heavily glycosylated. Several of the genes are subject to alternative splicing, and various family members, notably CECAM1, are expressed on a wide variety of cells including epithelial cells. The Opa proteins of both the gonococcus and the meningococcus react with the IgV domain of the molecules irrespective of its state of glycosylation, but react only with human CECAMs and not with those of mice or rats.

A smaller number of Opa proteins recognize heparan sulfate proteoglycans (HSPGs). The heparan sulfate occurs mainly on the syndecan class of molecules of which four have been described and two of these (1 and 4) are expressed on epithelial cells. The syndecans are believed to act as receptors or coreceptors for interaction between cells and the extracellular matrix. There is also evidence that fibronectin and vitronectin may enhance the signals for ingestion into the cells by bridging the HSPG recognized by the Opa protein to integrin $\alpha_v\beta_5$ or $\alpha_v\beta_3$ on the cell surface.

LPS

Like other Gram-negative bacteria, the pathogenic *Neisseria* carry LPS in the external leaflet of their outer membranes. In contrast to the high molecular weight LPS molecules with repeating O-chains seen in many enteric bacteria, the LPS of *Neisseria* is of modest size and therefore is often referred to as lipooligosaccharide (LOS). Although the molecular size of the LPS is similar to that seen in rough LPS mutants of *Salmonella* spp., this substance has considerable antigenic diversity. In the case of the meningococcus, a serological typing scheme has been developed that separates strains into 12 immunotypes, and the detailed structure of these has been determined. The LPS of the pathogenic *Neisseria* is heterogeneous, and LPS preparations frequently contain several closely spaced bands as analyzed by SDS–PAGE. Using monoclonal antibodies, it is evident that gonococci are able to change the serological characteristics of the LPS they express and that this antigenic variation occurs at a frequency of 10^{-2} to 10^{-3} per cell division.

The structure of the largest fully characterized gonococcal LPS molecule is shown in **Figure 1**. To the lipid A are linked two units of keto-deoxyoctulosonic acid (KDO) and two heptoses (Hep). This inner core region as shown in **Figure 1** can carry three oligosaccharide extensions that have been named the α-, β-, and γ-chains. The γ-chain consisting of *N*-acetyl glucosamine (GlcNAc) appears to be always present. The β-chain when present consists of a lactosyl group and when absent the position is substituted with ethanolamine phosphate. The gonococcal α-chain in its full form consists of the pentasaccharide, shown in **Figure 1**. An alternative α-chain structure consisting of a trisaccharide is also

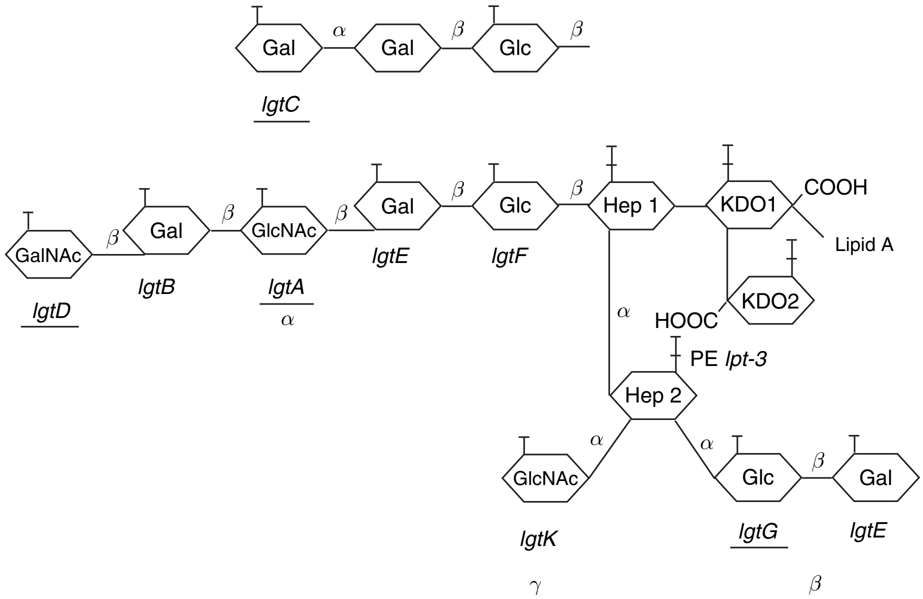

Figure 1 Genetics of neisserial lipopolysaccharide (LPS) synthesis. The LPS contains a lipid A portion with two residues of keto-deoxyoctulosonic acid (KDO) and linked to these are two residues of heptose (Hep) to form the inner core. This structure can bear three additional extensions indicated as the α-, β-, and γ-chains. The largest structurally characterized α-chain is indicated in the figure and consists of glucose (Glc), galactose (Gal), *N*-acetyl glucosamine (GlcNAc), Gal, and *N*-acetyl galactosamine (GalNAc). An alternative α-chain has been characterized and is the trisaccharide shown at the top of the figure. The glycosyltransferases responsible for the addition of each of the sugars are indicated by their genetic designation. The genes that are underlined are subject to high-frequency variation. The meningococcus lacks *lgtD* and hence does not have a terminal GalNAC residue. Gonococci and meningococci expressing the lacto-*N*-neotetraose α-chain grown *in vivo*, or *in vitro* in medium supplemented with cytosine monophosphate-*N*-acetyl neuraminic acid (CMP-NANA), will add a sialic acid residue. Among meningococcal strains, there is substantial variation in the sequence of the *lgtE* gene with two main evolutionary lines that are mutually exclusive, one of which is referred to as *lgtH*; they however perform the same biosynthetic task. Phosphoethanolamine (PE) is added to Hep 2 at carbon 6 by *lpt-3*. If the *lgtG* is inactive then *lpt-3* adds phosphoethanolamine at carbon 2 as well.

Table 4 Molecular mimicry by gonococcal lipopolysaccharide

Human antigen mimicked	*α-Chain oligosaccharide*
Lactosyl ceramide	Galβ1 → 4Glcβ1 → 4-R
Globoside, p^k blood group antigen	Galα1 → 4Galβ1 → 4Glcβ1 → 4-R
Lacto-*N*-neotetraose, paragloboside	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4-R
Gangliosides, X_2 blood group antigen	GalNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4-R
Sialyl-gangliosides	NANAα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 4-R

shown. However, as indicated in **Table 4**, the sugar composition of the α-chain can vary, and in every instance, it is identical to human cell surface oligosaccharides most often part of glycosphingolipids that in some instances are the determinants of blood group antigens. Meningococci lack *lgtD* the glycosyltransferase adding the terminal *N*-acetyl galactosamine.

The glycosyltransferases responsible for the biosynthesis of gonococcal and meningococcal LPS have been identified and are shown in **Figure 1**. This has provided an understanding of the genetic mechanism that underlies the high-frequency variation in the LPS structures expressed by these organisms. Note that four of these genes (*lgtA, lgtC, lgtD*, and *lgtG*) are underlined to indicate that they contain in their coding frames homopolymeric tracts of nucleotides. In the case of *lgtA, lgtC*, and *lgtD*, these are stretches of 8 to about 20 deoxyguanosines (poly-G), which can vary in size due to errors resulting from slipped strand mispairing during replication. In *lgtG*, there is homopolymeric poly-C tract. When the number of bases in the tracts is such that the coding frames are not disrupted, the respective glycosyltransferases are produced, but if the number changes, premature termination occurs and no functional enzyme is produced. Thus, the presence of the β-chain depends on whether functional LgtG glucosyl transferase is produced. If not then *lpt-3* substitutes the position with phosphoryl ethanolamine. Similarly, in the instance of the α-chain synthesis if *lgtA* is 'on', then the lacto-*N*-neotetraose chain will be formed and the addition of the terminal GalNAc depends on whether *lgtD* is 'on' or 'off'. When *lgtA* is 'off', then the globoside structure is synthesized if *lgtC* is 'on' and only the lactosyl structure if *lgtC* is 'off'. The gonococcus and the meningococcus have evolved a very elegant system to shift readily between a large number of different LPS structures, all of them being mimics of human glycolipids. This ability to shift the expression among a number of different LPS structures is not peculiar to the pathogenic *Neisseria*, but also occurs in *H. influenzae* whereat least four genes are subject to phase variation. In this organism, the mechanism is also by slipped strand mispairing but occurs in repeated tetrameric sequences that can be either CAAT or GCAA. Thus, it is likely that LPS antigenic variation is important since it is an attribute of a number of mucosal pathogens.

Gonococci possess a sialyl transferase activity which *in vitro* is able to use exogenously supplied cytosine monophosphate-*N*-acetyl neuraminic acid (CMP-NANA) and add NANA to the LPS if the organism is expressing the lacto-*N*-neotetraose α-chain (see **Table 4**). In the human infection *in vivo*, the concentration of CMP-NANA found in various host environments is sufficient to support this reaction. The sialylation of the LPS causes gonococci to become resistant to the antibody-complement-dependent bactericidal effect of serum. The resistance is to the bactericidal effect mediated not only by antibodies to LPS, but also to other surface antigens as well. Group B, C, W-135, and Y meningococci have the capacity to synthesize CMP-NANA as a precursor of their capsule biosynthesis and frequently sialylate their LPS without requiring exogenous CMP-NANA.

It is still unknown how this molecular mimicry illustrated in **Table 4** benefits the organism. It has been proposed that the human host may find it difficult to produce antibodies to any of these structures and that the ability to change to a different one may compound this problem. While immune evasion is attractive as an idea, it is clear that the LPS does serve as target for bactericidal antibodies, and at least *in vitro* perhaps the majority of bactericidal antibodies are directed to this antigen, rather than other surface structures. *In vivo* this is of course very different because sialylation of the LPS very effectively inhibits the bactericidal reaction and interferes with phagocytosis as well. However, only the lacto-*N*-neotetraose structure is effectively sialylated to produce the serum-resistant phenotype. (The exception is the L1 immunotype meningococcal LPS that has a glycosyltransferase capable of sialylating the globoside trisaccharide.) Why does the organism then have genetic mechanism to alter away from this structure? Perhaps the answer lies in the observation that sialylation of the LPS interferes with invasion of epithelial cells *in vitro*. There is also evidence that sialylated gonococci are significantly less infectious when used to challenge volunteers. It is clear that the gonococcus can circumvent LPS sialylation either by addition of the terminal *N*-acetyl galactosamine or by truncation of the chain. It is also possible that the mimicry may benefit the organism by allowing it to be recognized by human carbohydrate-binding molecules such as the C-lectins, the S-lectins, and the sialoadhesins.

Classification of Meningococci

Because of the central importance of useful classification systems to analyze the epidemiology of meningococcal endemic and epidemic disease, enormous effort has been invested in this endeavor. The first systems depended on the serological identification of the capsular serogroup, and this remains essential in making decisions concerning the use of polysaccharide-based vaccines.

However, as it became evident that the group B polysaccharide antigen was not suitable as a vaccine candidate (see below), serological classification schemes based on subcapsular antigens were developed in a number of laboratories. While at first these were based on empiric results obtained by bactericidal assays or gel diffusion studies, subsequently it became evident that these classifications were based primarily on epitopes on Por proteins or LPS determinants and this allowed the refinement of the classification often to a molecular description. During this time, a number of laboratories became interested in the study of bacterial population genetics and typing schemes based on the analysis of polymorphic housekeeping genes. Initially, this was accomplished by electrophoretic analysis of a number of enzymes (in the order of 12 to 15 or more) by so-called multilocus enzyme electrophoresis (MLEE). While there were a great number of different electrophoretic types (ET), it was observed that a few of these were disproportionately represented in the strains studied and also tended to exist in strains isolated from patients with disease in different areas of the world and at different periods of time. These were referred to as hypervirulent clones and the first so recognized were the ET-5 and ET37 complex. MLEE as a technique is quite demanding and interlaboratory reproducibility was difficult to achieve. Concurrently, the meningococcal genome sequence became available and DNA sequencing became a widely available technology. Thus, at this time MLEE has for the most part been replaced by multilocus sequence typing (MLST). Because of the greater number of alleles that can be recognized by sequence variability in a polymorphic enzyme, it has been possible to obtain the same resolution using only seven enzymes. The global types identified by this method are referred to as STs and overlap closely with the ones previously identified by MLEE, as summarized in **Table 5**.

Natural Immunity

Bactericidal Antibody

In the case of the meningococcus, there is clear evidence that the major predisposing factor for bloodstream invasion is the lack of biologically active antibodies to surface components and resultant failure to mediate an

Table 5 Prevalent multilocus sequence typing meningococcal complexes

ST complex	*Epidemiologic characteristics*
ST-41/44 complex lineage 3	New Zealand epidemic
ST-11 complex ET-37 complex	Mostly group C, UK problem, also African W-135
ST-32 complex ET-5 complex	Mainly group B worldwide since 1970
ST-8 complex cluster A4	B & C worldwide
ST-269 complex	Group B in Quebec
ST-213 complex	
ST-22 complex	
ST-23 complex	Group Y
ST-162 complex	

antibody-complement bacteriolytic reaction. This was first demonstrated in 1969 by Goldschneider and his colleagues by two lines of evidence. The first is based on a study done in an adult population. Nearly 15 000 sera were collected from military recruits within the first week of training and stored in anticipation that a number of these would develop meningococcal meningitis during the 8-week basic training. In fact 60 cases occurred in this cohort, and in 54 of these the *N. meningitidis* causing the infection could be isolated. Each of these sera as well as ten sera obtained from unaffected recruits serving in the same training platoons were tested for bactericidal activity against the strain of *N. meningitidis* isolated from the patient. Only 5.6% of the patients' sera were able to kill the disease-causing *N. meningitidis*, while 82% of sera obtained from unaffected recruits demonstrated bactericidal activity. The second line of evidence is the demonstration that there is an inverse relationship of the incidence of meningococcal disease and the prevalence of bactericidal antibody in relationship to age. The disease is very rare during the first 3 months of life when maternally derived antibodies are still present. Incidence rises to a peak between 6 and 12 months of age when the nadir of bactericidal activity is seen. Thereafter, the incidence progressively diminishes as the prevalence of antibodies rises with age. This is the same relationship that was reported for *H. influenzae* meningitis by Fothergill and Wright in 1933. Finally, it is evident that the antibody-complement-dependent bactericidal reaction is clearly important in protection against neisserial systemic infections since deficiencies of late complement components (C6 or C8) impart a specific susceptibility to blood-borne neisserial infections, but not to other bacterial infections.

Is there natural immunity to gonococcal infection? It is established that individuals with no known immunological defective can acquire gonorrhea multiple times. Thus, it has been suggested that there is no such thing as natural immunity to this disease. However, there is clear evidence that gonococcal infection before the days of antibiotic therapy was as a rule a self-limited disease lasting for a few weeks. This spontaneous elimination of the infection applied not only to the genitourinary disease, but often also to disseminated gonococcal infection, to gonococcal arthritis (albeit with bad sequelae), and even in some instances to gonococcal endocarditis. Hence, there is ample evidence that after a period of time gonococci are killed effectively *in vivo*. In the face of this ability to self-cure, how can we explain the apparent lack of natural immunity? The most likely explanation is that gonococci are inherently so antigenically variable that it requires the immune system considerable time to catch up with the repertoire of the infecting gonococcus and eliminate the infection.

Prevention

Two methods exist for the prevention of meningococcal disease: chemoprophylaxis and vaccination.

Chemoprophylaxis

Meningococcal disease represented a very serious problem to military forces during the major mobilizations of World War I and World War II. The disease often reached epidemic proportions in recruit camps. In 1944, it was demonstrated that these outbreaks could be prevented if all military personnel in a camp were treated with a brief course of sulfa drugs. This was highly effective in lowering the carrier rate and consequently the incidence of disease. This prophylaxis continued to control this problem until 1964 during the Vietnam mobilization when the meningococci circulating in the recruit camps had for the most part become sulfa-resistant. Since the introduction of effective meningococcal vaccines, the role of chemoprophylaxis has become restricted to prevention of disease among close contacts of cases. Close contacts include family members and members of the health care team that were most closely involved in the care of the patient. The agents used are rifampin, ciprofloxacin, and ceftriaxone.

Vaccines

Since the beginning of the twentieth century, attempts have been made to prepare vaccines for the prevention of meningococcal disease. Vaccines based on whole-cell preparations proved to be ineffective. In the late 1960s, methods were developed to purify the capsular polysaccharides of group A and C meningococcus in a form that maintained their high molecular weight. It was shown that injection of 25–50 μg of group A, C, Y, or W-135 polysaccharide in school-age children and adults resulted in a strong and long-lasting antibody response and that *in vitro* these antibodies were opsonic and bactericidal. Large-scale field trials both in the United States and overseas demonstrated that both group A and group C polysaccharide vaccines were highly effective in preventing the disease and that the protection lasted for at least 2 years. These vaccines were introduced in the US military over 30 years ago and have essentially eliminated the problem of meningococcal disease among recruits. Vaccination is employed in the military of many other countries and is required for pilgrims participating in the Hajj. In Egypt, meningococcal A/C vaccine is routinely given to children at school entry (age 6 years) and a second dose 3 years later, and since the introduction of this practice, the prevalence of meningitis has markedly changed for the better.

As a general rule, the immune response to purified polysaccharides is age related, but the response varies with the antigen. Thus, responses to the group C antigen are very low below the age of 18 months. Children between 2 and 4 years of age do respond to the group C antigen, but the response is short-lived lasting only a few months. After age 6, the responses are similar to adults. The response to group A antigen among young children is unusually favorable. Infants who are vaccinated twice, at 3 months and again at 6 months of age, will show a brisk booster immune response to the second injection which is sufficient to provide protection. This booster response has not been seen with any other polysaccharide antigen. It has been demonstrated that a protective level of group A antibodies can be maintained by immunization twice in the first year of life, then again at age 2 and upon entry to school. Unfortunately, this property of the group A antigen has not been taken advantage of in the prevention of endemic and epidemic disease in Africa.

As the experience with the *H. influenzae* vaccine has demonstrated, the immune responses in infants and toddlers can be markedly improved by covalently linking the polysaccharide to a protein carrier to enhance T cell help in the immune response. In 1999, England introduced vaccination with group C meningococcal conjugate vaccine in infants, with a three-dose schedule at 2, 3, and 4 months. In addition, over a 1-year period, a catch-up campaign reached 85% of children 5–12 months of age and teenagers 15–17 years with a single dose. The effectiveness over a 1-year period was over 90%. There was also a 67% reduction of group C disease among the unvaccinated population, evidence of herd immunity attributable to the drop in the group C carrier rate in the age group 15–17 years. However, in the infants receiving the vaccinations, effectiveness diminished after 1 year following the last immunization. This decline of effectiveness has also been seen with the Hib conjugate vaccine and has led in the United Kingdom to the practice of giving a booster at the age of 12 months. In Spain, routine vaccination was introduced in 2000, with a three-shot schedule at 2, 4, and 6 months. Effectiveness in the first year was 98%, but declined to 78% in the subsequent year. Development of conjugate vaccines has progressed rapidly, and the first quadrivalent vaccine (A, C, W-135, and Y) was FDA-approved in 2005.

The group B capsular polysaccharide is a homopolymer of $\alpha(2\text{–}8)$-linked NANA (see **Table 2**). This structure is present on mammalian tissues notably on neural cell adhesion molecule (N-CAM), and the degree of $\alpha(2\text{–}8)$ sialylation of this molecule is particularly elevated during embryonic life. While the majority of adults have some levels of antibodies to this antigen, injection of the purified antigen generally does not raise any additional antibodies. There has also been concern that engendering a strong immune response to this antigen may have deleterious effects on infants during fetal life. Therefore, group B meningococcal vaccines based on partially purified outer membranes with their LPS content reduced by detergent extraction have been prepared and have proved to be able to prevent disease caused by the strain used for the production of the vaccine. They have proved efficacious under epidemic conditions in Cuba, in Norway, and most recently in New Zealand, showing up to 80% protection against the epidemic strain prevailing in the respective country. However, they have not provided broad protection against group B strains because of the considerable antigenic heterogeneity in meningococcal outer membrane proteins.

Recently, a new approach has been adopted for this problem, which is now referred to as reverse vaccinology. Its essence is to use available genomic sequences to predict protein antigens that are likely to be surface exposed. These are then produced by recombinant DNA methodology and tested for their ability to give rise to protective antibodies in mice. Nearly 100 proteins not previously described were expressed, among which a quarter gave rise to bactericidal antibodies. With the aid of the MLST system described above, it is now possible to assemble strain collections that are representative of group B hypervirulent clones seen worldwide over the last decades in order to test the broadness of potential protection that can be induced by these antigens either singly or in combination. Based on the bactericidal response elicited in mice by immunized with a combination of a particular set of five antigens presented in a vaccine formulation suitable for use in humans is predicted to afford protection against 80% of group B strains currently in circulation. These immune responses if they extend to human beings may lead to a practical vaccine to prevent group B meningococcal disease.

No vaccine exists at this time for the prevention of gonorrhea, and the problem is formidable because of the extraordinary antigenic variability of this organism. Nevertheless, the experience in several European countries has demonstrated that prevention of this disease can be very effective if public education is combined with rapid treatment of infected individuals and their contacts. To invest in this approach is becoming increasingly important because of the formidable antibiotic resistance pattern seen in current strains and the role it plays in heightening the transmissibility of HIV-1 infection.

Conclusion

The discrete steps that occur in the mucosal infection by the pathogenic *Neisseria* are increasingly being explained on molecular cell biological level. It is evident that particularly the gonococcus has developed very elaborate mechanisms to evade the immune response of human beings shared to a lesser extent by the meningococcus. With pili, it has chosen the path of antigenic variation. This is an evasion mechanism which is highly developed in eukaryotic parasites such as trypanosomes and is also seen in prokaryotes such as *Borrelia.* In the case of Rmp, the gonococcus has chosen the path of antigenic constancy as a target for blocking antibodies. With Opa proteins, the variation may be more a way to succeed in various environments within the host rather than immune evasion. The biological significance of LPS variation is not yet clear, but it must be very useful since *Neisseria* and *H. influenzae* have developed in principle the same variation mechanism, though the specific details are quite different. In the era before ready treatment with antibiotics, self-cure of gonorrhea over a period of weeks was commonly seen, and this slow acquisition of natural immunity was probably a reflection of the time needed for the immune response to finally catch up with the variability of the particular strain infecting the human host. In the case of the meningococcus, the introduction of the polysaccharide conjugate vaccines will have an enormous impact on this disease since it allows the extension of benefits of vaccination to infants and young children, the population at greatest risk, and there is every reason to expect that the impact on disease incidence will be just as profound as it has been with hemophilus type b meningitis. There is also increasing optimism about the possibility to develop a group B meningococcal vaccine based on a carefully selected combination of protein antigens.

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Helicobacter Pylori

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Glossary

chronic active gastritis The infiltration of the gastric mucosa with lymphocytes, plasma cells, and granulocytes. Essentially, all people colonized with *Helicobacter pylori* develop chronic active gastritis, but the intensity varies.

Helicobacter pylori *Helicobacter pylori* is one of the most common bacteria affecting humans, colonizing more than half of the world's population. *H. pylori* colonizes the gastric mucosa, frequently persisting for the entire life of the host. This colonization invariably causes a chronic tissue response of the gastric mucosa, termed chronic or chronic active gastritis. Although this process is asymptomatic, those colonized are at a higher risk of several illnesses including gastric and duodenal ulcer, gastric adenocarcinoma, and lymphoma involving the MALT. Interestingly, persons with *H. pylori* are at a lower risk for gastroesophageal reflux and its sequelae, and possibly asthma. *H. pylori* was first isolated in culture in 1982 and *H. pylori* research has since become one of the most rapidly moving fields in medical microbiology. Although the focus of this article is on data that are generally accepted, we have included a number of areas that remain controversial.

mucosa-associated lymphoid tissue (MALT) Tissue that frequently responds to *H. pylori* colonization. The 'germ-free' stomach does not have MALT. The presence of gastric MALT can give rise to malignant B cell lymphomas, which are rare events.

panmictic population structure A population structure that arises when recombination is so frequent in a given bacterial species that no remnants of clonal descent are discernible.

pathogenicity island (PAI) A large fragment of DNA in the genome of a microorganism that contains virulence-related genes and has been acquired by horizontal gene transfer. Hallmarks of PAI are GC content that differs from that of the rest of the genome, insertion in proximity to tRNA genes, and mobility genes (e.g., insertion sequences).

peptic gastric/duodenal ulcer A breach in the epithelium of the stomach or duodenum caused by an imbalance between aggressive factors (acid and pepsin) and mucosal protection mechanisms. Ulcers have a strong tendency to relapse and can progress to the potentially fatal complications of bleeding and perforation; removal of *H. pylori* colonization ameliorates ulcer disease.

urease An enzyme abundantly produced by *H. pylori* that hydrolyzes urea. It is essential for gastric colonization. Detection of urease activity is used to diagnose the presence of *H. pylori* by the rapid biopsy urease test and the ^{13}C urea breath tests.

vacuolating cytotoxin (VacA) A cytotoxic protein that affects epithelial and immune cell function. Multiple alleles exist that vary in VacA production *in vitro*. Colonization with particular genotypes affects the risk of disease development.

Abbreviations

GERD	gastroesophageal reflux disease
LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissue
NSAID	nonsteroid anti-inflammatory drug
VacA	vacuolating cytotoxin

Historical Introduction

Pathologists noted the presence of spiral bacterial in the human stomach as early as 1906. Although similar observations were repeatedly reported during the subsequent decades, they did not receive much attention because the bacteria could not be cultured. The introduction of flexible fiberoptic endoscopes and the establishment of microaerobic and selective culture techniques for *Campylobacter* species were important prerequisites for the first isolation in culture of *Helicobacter pylori* from human gastric biopsies by two Australian researchers, Barry Marshall and Robin Warren in 1982. The most important argument for a pathogenic role of *H. pylori* came from clinical trials showing that the elimination of *H. pylori* substantially changes the clinical course of ulcer disease. The elimination of *H. pylori* with antibiotic-containing regimens significantly reduced the high relapse rate of gastroduodenal ulcer disease. Thus, peptic ulcer disease, which previously could only be controlled by long-term treatment with inhibitors of gastric acid secretion or by surgery, became a condition that can be substantially improved by a short course of antibiotic treatment. In 2005, Marshall and Warren received the Nobel Prize in Medicine for their first isolation of *H. pylori* and showing the role of eradicative therapy in the control of ulcer disease. However, the long-term consequences of *H. pylori* eradication are not known.

In the early 1990s, three large prospective seroepidemiological studies each indicated that *H. pylori* is a major risk factor for the development of gastric noncardiac adenocarcinomas and *H. pylori* was classified as a definitive carcinogen by the World Health Organization in 1994. *H. pylori* also has been strongly associated with malignant non-Hodgkin's lymphomas of the stomach. In recent years, there has been increasing attention to its roles protecting against esophageal, pulmonary, and metabolic diseases.

Microbiology

General Microbiology

H. pylori was first designated *Campylobacter pyloridis*, then *Campylobacter pylori*; the new genus *Helicobacter* was established in 1989. It is a motile, urease-, catalase-, and oxidase-producing Gram-negative eubacterium that is classified in the epsilon subdivision of the proteobacteria on the basis of 16S rRNA sequence analysis. *H. pylori* requires rich media supplemented with blood, serum, or cyclodextrin and grows best at 37 °C in a microaerobic atmosphere (5% oxygen is optimal). Colonies become visible after 2–5 days of incubation. *H. pylori* cells *in vivo* are spiral-shaped (**Figure 1**), whereas after *in vitro* culture, cells with curved rod shape predominate. After prolonged incubation or after treatment of cultures with subinhibitory concentrations of certain antibiotics, *H. pylori* cells assume a coccoidal form. Whether these coccoid cells are in viable-but-non-culturable (VBMC) state and of epidemiological relevance, or are the morphological correlate of cell degeneration and death is still the subject of controversy. *H. pylori* cells have six to eight unipolar sheathed flagella.

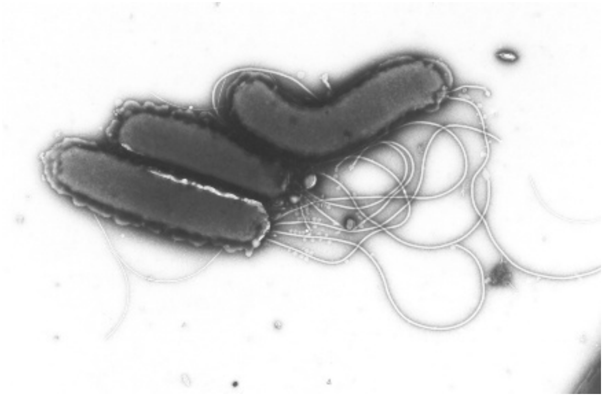

Figure 1 Electron micrograph (negative staining) of *Helicobacter pylori*, strain N6. Note the presence of sheathed flagella attached to one cell pole. At the tip of the flagella, the sheath forms a characteristic terminal bulb. The length of the bar corresponds to 0.5 μm. EM courtesy of Dr. Christine Josenhans (Hannover Medical School, Germany).

H. pylori is the type species of the genus *Helicobacter*, which comprises more than 25 species, with more being discovered every year. *Helicobacter* sp. colonize the gastric mucosa of particular mammals; some have been widely used in animal models of gastric *Helicobacter* colonization. The best studied among these are *Helicobacter mustelae*, which naturally colonizes ferrets, and *Helicobacter felis*, which in nature colonizes cats and dogs, but experimentally also colonizes mice. The term *Helicobacter heilmannii* is used for several – mostly as yet unculturable – *Helicobacter* species other than *H. pylori* with a distinct morphology (tightly and regularly coiled spirals) that are uncommonly found in the human stomach. The genus *Helicobacter* also contains nongastric species, such as the human enteric pathogens *Helicobacter cinaedi* and *Helicobacter fennelliae*, intestinal colonizers (*Helicobacter muridarum* and *Helicobacter pametensis*), and bile-resistant organisms that first were isolated from the biliary tract and the liver of rodents (*Helicobacter hepaticus* and *Helicobacter bilis*), but whose normal habitat may in fact be the colon (enterohepatic *Helicobacter* species). Some enterohepatic *Helicobacter* sp. (chiefly, *H. hepaticus*) can cause hepatobiliary cancer in immunocompetent mice as well as typhlitis, colitis, and colon cancer in immunodeficient mice.

Genome Sequence

H. pylori (strain 26695) has a circular chromosome of 1.668 Mb and an average GC content of 39%; the genome contains 1590 predicted coding sequences. Two other complete genome sequences (strains J99 and HPAG1) were reported more recently. Each of the genomes contains a substantial number of genes (approximately 100) that are not present in the other strains, most of them located in a highly variable chromosome region (the 'plasticity region'). A predicted 1111 genes are present in every strain, representing the *H. pylori* core genome. Most strains isolated from patients with symptomatic disease harbor a 37-kb pathogenicity island (PAI), the *cag* PAI. The genome is rich in homopolymeric tracts and dinucleotide repeats that permit phase variation of gene expression by frameshifts due to slipped-strand mispairing. Three fucosyl transferases involved in the synthesis of unusual oligosaccharides (Lewis^x, Lewis^y) in the lipopolysaccharide (LPS) O-specific side chains may undergo such phase variation. The Lewis antigens are identical to human gastric epithelial cell antigens and there is evidence for host selection of *H. pylori* Lewis phenotypic expression. Many *H. pylori* strains contain plasmids, whose biological functions are unknown. The genome sequence of the enterohepatic species *H. hepaticus* shares extensive homology with both gastric *Helicobacter* sp. and *Campylobacter* sp., and harbors a PAI, termed HHGI1.

Population Genetics and Evolution

H. pylori is highly diverse, as indicated by the unusually high sequence variation of both housekeeping and virulence-associated genes, as well as in variability of gene order. Much of this diversity is due to the unusual combination of a relatively high mutation rate, which is likely due to the lack of some DNA repair mechanisms, and very frequent intraspecific recombination during mixed infections of one human carrier with at least two strains, leading to the shuffling of alleles and individual mutations among different strains, and thus to the rapid disruption of clonal groupings. This high rate of recombination is, probably at least in part, due to the natural transformability exhibited by many strains of *H. pylori*. The population structure of *H. pylori* is largely panmictic, but different populations and subpopulations have been identified in *H. pylori* strains from different geographic regions. The geographic distribution of *H. pylori* populations mirrors ancient and more recent human migrations, and patterns of genetic diversity in *H. pylori* populations show strong similarity to patterns of genetic diversity in human DNA. *H. pylori* gene sequences have the potential to be even more informative about recent human migrations than human genetic markers. For unknown reasons, significantly different allelic types have been conserved at some gene loci, some of which are statistically associated with an elevated risk of developing disease (e.g., fragments of the *vacA* cytotoxin gene).

Colonization and Host Interaction Factors

H. pylori colonizes the gastric mucus layer and epithelium, a niche of the human body that normally has low levels of colonization by other bacteria. Humans and nonhuman primate species are the only known natural hosts of *H. pylori*; no reservoir in the inanimate environment has been identified. *H. pylori* has a strong tropism for the gastric epithelium. In the duodenum, it only colonizes areas where the normal duodenal mucosa has been replaced by gastric-type epithelium (gastric metaplasia). The colonized stomach contains two *H. pylori* subpopulations, the majority of bacteria moves freely in the viscous mucus layer that covers the gastric epithelium and others that are attached to gastric epithelial cells. The relative contributions to these subpopulations to persistence and tissue interaction are unknown. Intracellular bacteria are rarely observed, and *H. pylori* is considered an extracellular organism. Although a number of bacterial factors have been implied in the pathogenesis of *H. pylori*, there is little experimental evidence to support a specific pathogenetic role for many of these. Here, we review only those factors for which there is strong experimental or clinical evidence that supports their role in colonization or tissue injury: urease, motility, adherence, the vacuolating cytotoxin (VacA), and the *cag* island.

Urease

H. pylori produces large amounts of urease, a nickel-containing metalloenzyme that catalyzes the hydrolysis of urea. *H. pylori* urease is a hexapolymer composed of two subunits, UreA and UreB, present in a 1:1 stoichiometry, to which are bound two Ni^{2+} ions. Urease has multiple functions. In the presence of urea, urease permits *H. pylori* to maintain a constant internal and periplasmic pH, even when the external pH is strongly acidic, thereby preventing a collapse of the transmembrane potential difference. Isogenic urease-negative mutants of *H. pylori* are incapable of colonizing the gastric mucosa in several experimental challenge models in animals. This inability to colonize could not be overcome by blocking the host animal's acid secretion, indicating that protection of the bacteria from acid is not the sole function of urease. Urease may permit the use of urea as a nitrogen source and contribute to tissue injury by the generation of ammonia as well as by the recruitment and activation of inflammatory cells. Activity of urease is controlled by pH-dependent transcriptional regulation of urease gene expression as well as by specific transport mechanisms regulating the intracellular availability of the substrate urea and of the essential cofactor nickel.

Motility and chemotaxis

H. pylori is a highly motile bacterium, due to a bundle of six to eight unipolar flagella. Each flagellar filament is covered by a membraneous flagellar sheath, shielding the inner filament from low pH. The flagellar filaments of *H. pylori* are copolymers of two subunits, the flagellins FlaA and FlaB. Concomitant expression of both flagellin proteins is essential for full motility. In all, more than 60 genes are involved in the biogenesis of flagella, the assembly of the flagellar motor, and the chemotaxis system, and the expression of most of these is coordinately regulated by a complex regulatory network. Motility, as is urease, is essential for *H. pylori* to colonize its host. Mutants that are defective in the synthesis of either one of the two flagellins are severely impaired in colonization efficiency, whereas nonmotile double mutants are completely avirulent. An intact pH gradient across the mucus layer is required for the orientation of *H. pylori* within the mucus layer, where the bacteria reside in a narrow zone adjacent to the epithelial cells.

Adherence

H. pylori can adhere tightly to human gastric epithelial cells. More than 10 distinct *H. pylori* binding specificities for host cell glycoproteins, carbohydrates, and phospholipids have been reported. The best-characterized adhesins, BabA and SabA, are outer membrane proteins that mediate *H. pylori* binding to the Lewis^b and sialyl-Lewis^x tissue antigens, respectively. *bab*A and *sabA* are part of a large family of closely related genes encoding similar proteins (*Helicobacter* outer membrane proteins, Hop). The gene family encodes further adhesins (including AlpA, AlpB, and HopZ), whose receptors have not yet been characterized.

VacA

H. pylori produces a protein toxin that was first identified by its ability to induce vacuole formation in eukaryotic cells. The protein responsible for this vacuolization, VacA, first purified in 1992 by Cover and Blaser, is produced as a 140-kDa protoxin and actively secreted by means of a C-terminal autotransporter domain. The mature 87-kDa toxin present extracellularly forms multimeric complexes that resemble flowers with six or seven petals. VacA is a multifunctional toxin that acts on both epithelial cells and T cells. Several different activities have been reported, including the formation of anion-selective pores in cellular membranes, the induction of epithelial cell apoptosis by release of cytochrome *c* from mitochondria, inhibition of T cell proliferation, and interference with antigen presentation. The toxin gene occurs in several allelic forms, and a classification has been developed based on three polymorphic regions, termed signal sequence, middle region, and intermediate region (s/m/i regions). Allelic variants encode toxin molecules with different activity and receptor affinity. Generally, the m1/s1 form seems to be the one most strongly associated with disease. Although the extraordinary resistance of VacA to degradation by acid and pepsin, and its activation by acid indicate that it is highly adapted to an acidic environment, the *in vivo* role of VacA in human disease remains to be fully established.

CagA and the cag *PAI*

The *cag* PAI is a chromosomal region of >30 kb, encoding ~28 genes. Distribution of the *cag* PAI varies with geographic regions, about 70% of strains from Western countries contain the island, while *cag* PAI carriage is almost universal in Asia. In contrast, at least one population of *H. pylori*, termed hpAfrica2, exists in South Africa that always lacks a *cag* PAI. Possession by a strain of a functional *cag* PAI is associated with a higher risk of tissue response (greater numbers of inflammatory cells, more induction of proinflammatory cytokines, such as IL-8), and a higher risk of ulcers, mucosal atrophy, and gastric carcinoma. Several of the *cag* PAI genes encode proteins with homology to components of the T pilus of *Agrobacterium tumefaciens*, the prototype of a type IV secretion apparatus. Type IV secretion systems are multisubunit nanomachines that can introduce proteins (and/or DNA) into host cells and thereby influence cellular functions. After contact with host cells, using an integrin on the epithelial cell surface as a receptor, the *cag* type IV apparatus forms a pilus-like appendage that translocates the protein CagA into host epithelial cells. After its delivery into the host cell, CagA becomes phosphorylated by cellular kinases at specific phosphorylation sites (EPIYA motifs), and binds to several target proteins, including SHP-2, Csk, and PAR-1. These interactions, of which some are phosphorylation-dependent and some are phosphorylation-independent, induce multiple events that contribute to cellular responses, such as the morphogenetic changes characteristic of cell infection with *cag*-positive *H. pylori* strains, and may ultimately lead to malignant transformation. CagA has therefore been termed a bacterial oncoprotein. About 70% of all *H. pylori* strains possess the *cag* PAI.

Epidemiology

H. pylori occurs in all parts of the world and a large body of evidence indicates that colonization has been present since ancient times, for at least 50 000 years, if not considerably longer. The major determinants of prevalence are socioeconomic conditions and age. In developing countries, nearly everyone acquire *H. pylori* by age 10, but in developed countries, the overall prevalence is 40–50%. In industrialized countries, the incidence of *H. pylori* acquisition has been decreasing rapidly, probably due to improved hygienic conditions and widespread antibiotic use. *H. pylori* is mostly acquired in childhood. The route of acquisition and infectious dose are unknown; there is evidence for fecal–oral, oral–oral, and vomitus–oral transmission. Contaminated water supplies also may contribute to transmission. Direct transmission from person to person occurs within families, between spouses, and in communities where people live together in close contact, such as orphanages. The household is the most important place for transmission. Young children may be the most important amplifiers for transmission of the organisms. *H. pylori* is equally frequent in women and men. Twin studies have shown that susceptibility to *H. pylori* contains a hereditary component, which may be particularly relevant as it is disappearing rapidly.

Diseases Associated with *H. pylori*

Acute and Chronic Active Gastritis

The acute acquisition of *H. pylori* is rarely diagnosed. In most cases, it is characterized by some, nondiagnostic, abdominal symptoms, such as dyspepsia, abdominal cramping, and vomiting. Essentially all colonized persons develop a tissue response that is termed chronic active gastritis, characterized by the infiltration of the gastric mucosa with lymphocytes and plasma cells (chronic component) as well as neutrophils (active component). This response varies substantially in its intensity and anatomic distribution. However, most investigators believe that it is the nature of the specific response that affects clinical consequences, such as the risk of ulcer disease or cancer. *H. pylori* colonization has complex effects on gastric physiology (gastrin, somatostatin, leptin, and ghrelin secretion, and on acid secretion) that probably are dependent on both bacterial and host factors.

Ulcer Disease

Peptic ulcers of the stomach or the duodenum are common and potentially fatal conditions. Duodenal ulcers are usually associated with *H. pylori*, although medication (nonsteroid anti-inflammatory drugs (NSAIDs))-associated ulcers are becoming more common. Elimination of *H. pylori* substantially reduces ulcer relapses. The same holds true for gastric ulcers, although NSAID induction is proportionally more common. It is in the treatment of ulcer disease that the discovery of *H. pylori* has had the most significant clinical impact. The recommendation is for *H. pylori* to be eliminated in all patients with ulcer disease.

Gastric Carcinoma

Large seroepidemiological studies have shown that the presence of *H. pylori* increases between three- and ninefold the risk for subsequent development of noncardia gastric adenocarcinoma, and experimental infection of Mongolian gerbils confirms the oncogenic role. About 60% of all gastric cancers, or 500 000 new cases per year, can be attributed to the presence of *H. pylori*. Carriage of *cagA*+ strains induces higher risk of both ulcer disease and gastric cancer.

Gastric Lymphoma

Gastric B cell non-Hodgkin's lymphomas are relatively rare gastric malignancies (about 1 per 1 million in the population annually) that in most cases arise from acquired mucosa-associated lymphoid tissue (MALT). *H. pylori* almost always induces the development of lymphoid follicles in the submucosa; in the absence of *H. pylori*, the stomach is usually devoid of MALT. Gastric MALT lymphomas are therefore very rare in patients without *H. pylori*. In low-grade MALT lymphoma, the proliferation of the malignant B cell clones appears to be dependent on stimulation by bacterial antigens, probably explaining why *H. pylori* eradication may induce tumor remissions. The boundary between benign hyperproliferation and true malignancy is not fully resolved.

Esophageal Diseases

In the twentieth century, as *H. pylori* prevalence has dropped in developed countries, the incidence of esophageal diseases such as gastroesophageal reflux disease (GERD) and its sequelae, including Barrett's esophagus and adenocarcinoma of the esophagus, has increased dramatically, and the trend continues. Is there any relationship between the decline in *H. pylori* and the rise of these diseases? A large body of evidence now indicates that persons carrying *H. pylori*, especially *cag*+ strains, have a substantially lower risk of GERD and its sequelae, including adenocarcinoma, than persons who are *H. pylori*-free. The secular trend, pathophysiologic, and epidemiologic studies all point toward gastric *H. pylori* colonization as protecting the esophagus from these diseases.

Asthma and Allergic Diseases

Similar to GERD, these diseases also have been increasing as *H. pylori* is disappearing from developed country populations. Several large studies now have shown an inverse relationship between *cagA*+ *H. pylori* strains and asthma, allergic rhinitis, eczema, and skin sensitization, especially for cases with childhood onset. If these findings are confirmed, they will substantially change our evaluation of the clinical significance of *H. pylori.*

Diagnosis

Diagnosis of *H. pylori* can be established by gastric biopsy obtained during an endoscopy of the upper gastrointestinal tract, as well as by noninvasive techniques. The simplest biopsy-based test for *H. pylori* is the rapid urease test, which detects the abundant urease activity. The bacteria also can be visualized in tissue sections when specific histological staining techniques (e.g., the Warthin–Starry stain) are used. Culturing *H. pylori* is rarely done in clinical settings, but may be advisable when determination of antibiotic susceptibility is important. Essentially all colonized people develop humoral (especially serum IgG) immune responses to *H. pylori* antigens, a property that can be used to diagnose its presence without endoscopy. An alternative way to diagnose *H. pylori* presence is by the ^{13}C urea breath test. Stool antigen tests also can be useful, especially in children.

Treatment

The recommendation is to eliminate *H. pylori* in all patients with gastric or duodenal ulcer disease, with low-grade gastric MALT lymphoma, or who have undergone surgery for resection of early-stage gastric carcinoma. Although *H. pylori* is susceptible to many antibiotics *in vitro*, no single agent achieves eradication rates above 55%, and thus combination therapies must be used. The standard treatment to eliminate *H. pylori* in 2008 consists of a combination of two antibiotics (most commonly the macrolide clarithromycin, in combination with either a nitroimidazole or amoxicillin) with an inhibitor of gastric acid secretion (such as a proton-pump inhibitor), which are given for 7–10 days. These short-term regimens have low rates of side effects, and in clinical practice achieve eradication rates exceeding 80%. Increasing *H. pylori* resistance to nitroimidazoles and macrolides has reduced their efficacy; this resistance is due to the acquisition of point mutations in the nitroreductase gene *rdxA* and 23S ribosomal RNA genes, respectively.

Vaccine Development

When *H. pylori* was considered to be exclusively a pathogen, vaccine development appeared to be desirable. Accumulating evidence suggesting a protective role against esophageal diseases and other disorders has tempered the recommendations concerning worldwide elimination of these organisms. Vaccine strategies in the future may be aimed at particular populations at high risk of gastric cancer, or in preserving colonization, but reducing the specific disease risks.

Conclusions

The isolation of *H. pylori* and analysis of its relation to disease has had an important impact on the clinical practice of medicine, a phenomenon that continues to evolve. Because peptic ulcer disease had been considered for so long to be a medical disease without the consideration of a microbial role, the elucidation of the contributions of *H. pylori* also has reinvigorated the search for microbial participation in other diseases whose pathogenesis is at present poorly understood (e.g., inflammatory bowel disease, atherosclerosis, and biliary tract diseases). The paradigm of *H. pylori* protecting against potentially fatal conditions provides a new dimension that needs to be taken into account when strategies for management of *H. pylori* infection are devised. The comparative assessment of benefits (e.g., potentially preventing an excess of 500 000 cases of gastric cancer per year) and costs of mass *H. pylori* eradication and prevention is far from being completed. As our understanding of the interaction of *H. pylori* with humans matures, it provides answers to ecological questions about relationships with our commensal organisms.

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Hepatitis Viruses

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Glossary

alanine transaminase (ALT, formerly SGPT) An enzyme found predominantly in liver, released into the serum as a result of liver injury. In most liver diseases, ALT is elevated to a greater level than the aspartate transaminase (AST), and in acute viral hepatitis levels of >1000 IU ml^{-1} are not uncommon.

alkaline phosphatase (ALP) An enzyme that is made in the liver, bone, and the placenta and can be measured in serum. It is increased in the setting of diseases in which bile secretion is impaired, and to a lesser degree in association with hepatocellular injury.

angioedema A skin eruption similar to urticaria but affecting both the dermis and the subcutaneous tissue (deeper skin layer).

ascites Abnormal accumulation of fluid in the abdominal cavity. Ascites is associated with advanced liver disease complicated by portal hypertension but can be seen in other conditions including abdominal malignancies.

aspartate transaminase (AST, formerly SGOT) An enzyme found in many tissues including the liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged, for example, after a myocardial infarction or muscle injury. Although not a highly specific indicator of liver injury, levels of >500 IU ml^{-1} suggest acute hepatitis.

B cells These lymphocytes mature in the bone marrow and are responsible for humoral immunity.

bilirubin A chemical that results from the breakdown of red blood cells. Increased levels in serum result from increased red cell destruction (hemolysis), decreased uptake by the liver, or decreased excretion into the bile. High levels in the serum are associated with jaundice.

cholestasis Caused by obstruction to bile flow within the liver (intrahepatic) or outside the liver (extrahepatic). The obstruction causes bile salts, the bile pigment bilirubin, and fats (lipids) to accumulate in the blood instead of being eliminated normally.

cirrhosis The final common histological pathway for a wide range of chronic liver diseases, the hallmark of which is the formation of microscopic or macroscopic nodules of normal liver tissue separated by bands of fibrous tissue. Injury to the liver cells results in an inflammatory response that leads to the formation of scar or fibrous tissue. The liver cells that do not die replicate to replace the cells that have died, resulting in clusters of newly formed liver cells (regenerative nodules) within the fibrous tissue.

cytopathic Pertaining to or characterized by pathological changes in cells.

cytopenias A reduction in the number of cells circulating in the blood, which can take several forms; anemia is a decrease in red cell count, leucopenia is a decrease in white cells, and thrombocytopenia is a reduction in platelets. When all three classes of blood cells are decreased, the condition is known as pancytopenia.

cytotoxic T lymphocytes Lymphocytes that mature in the thymus gland and generate cell-mediated immune responses, directly destroying cells that have specific antigens on their surface recognizable by these T cells.

deoxyribonucleic acid (DNA) The genetic material of all cellular organisms and most viruses. A molecule of DNA consists of two strands composed of nucleotides, linked together to form a chain in the form of a double helix. DNA carries the information needed to direct protein synthesis and replication.

enteral Entry of a substance or organism (such as a virus) into the body via the gastrointestinal tract.

epitope Also known as the antigenic determinant, this is the specific part of an antigen that can be recognized by an antibody.

glomerulonephritis An inflammation of the glomeruli, bunches of tiny blood vessels inside the kidneys. The damaged glomeruli cannot effectively filter waste products and excess water from the bloodstream to make urine.

HBV cccDNA Hepatitis B virus covalently closed circular DNA is a continuous double-chain ring, which serves as the template for all viral RNA transcription.

hepatic portal system A portal system is a capillary bed draining into another capillary system through veins before returning to the heart. The hepatic portal system refers to the circulation of blood from parts of the gastrointestinal tract, via the hepatic portal vein into the hepatic sinusoids.

hepatic steatosis One of a spectrum of liver diseases termed nonalcoholic fatty liver disease (NAFLD) that have in common the accumulation of excess fat in liver cells. These conditions range from simple steatosis (excess fat without inflammation), to nonalcoholic steatohepatitis (NASH), through to cirrhosis. The term nonalcoholic is used because NAFLD occurs in individuals who do not consume excessive amounts of alcohol, but the appearance of the liver microscopically is similar to that seen in alcoholic liver disease. NAFLD is associated with insulin resistance and the metabolic syndrome.

hepatomegaly Enlargement of the liver.

humoral immune response The production of antibodies that circulate through the blood and other body fluids, binding to antigens, and helping to destroy them.

insulin resistance (IR) The uptake of glucose into tissues is stimulated by insulin, in the setting of IR, tissues have an impaired responsiveness to the actions of insulin. In an effort to maintain normal glucose levels, the pancreas secretes more insulin, leading to high plasma insulin levels. Over time the pancreas is unable to overcome IR through hypersecretion and overt diabetes develops.

International Normalized Ratio (INR)/prothrombin time These laboratory tests are measures of the extrinsic pathway of coagulation, and are a sensitive method of assessing liver function. The INR is the ratio of a patient’s prothrombin time to a normal (control) sample.

jaundice The yellowish coloration of the skin and sclerae (the whites of the eyes) observed when bilirubin levels are increased above a certain level. Also referred to as icterus.

lipoprotein Classes of conjugated proteins in which proteins are combined with a lipid (fat) such as cholesterol. These complexes are the form in which lipids are transported in the circulation. Lipoproteins are classified by their density and chemical properties.

lymphocyte Specialized white blood cells whose function is to identify and destroy invading antigens, are subdivided into B and T cells.

major histocompatibility complex (MHC) A large cluster of genes located on the short arm of chromosome 6, which is traditionally divided into the class I, II, and II regions, each containing groups of genes with related functions. Many, but not all of the genes in this complex play important roles in the immune system.

metabolic syndrome This syndrome can be defined as a number of related conditions, including obesity, hypertension, abnormalities of lipid metabolism, and type 2 diabetes, that are associated with IR and compensatory hyperinsulinemia.

parenteral Entry of a substance or organism (such as a virus) directly into the bloodstream, via a device such as needle or catheter.

polyarteritis nodosa A disease of unknown etiology, possibly due to hypersensitivity to an unknown antigen, causing inflammation and necrosis of medium-sized muscular arteries, with secondary ischemia of tissue supplied by affected vessels.

portal hypertension Elevated blood pressure in the portal vein and its branches, resulting from intrahepatic or extrahepatic portal venous compression or occlusion. In the United States and Europe, the commonest cause is increased resistance to blood flow caused by extensive scarring of the liver in cirrhosis. Increased pressure in the portal circulation causes the formation of new veins called collaterals that develop at specific places, most importantly at the lower end of the esophagus and upper part (fundus) of the stomach.

purpura Hemorrhages in the skin and mucous membranes having the appearance of purplish spots or patches.

ribonucleic acid (RNA) A molecule of nucleic acid that differs from DNA by containing ribose rather than deoxyribose. RNA is formed on a DNA template. Several differing molecular classes of RNA are produced (messenger, transfer, and ribosomal) that play roles in the synthesis of protein and other cell functions. It reflects the exact nucleoside sequence of the genetically active DNA.

splenomegaly Enlargement of the spleen.

urticaria A skin eruption consisting of localized wheals and erythema (‘hives’) affecting only the dermis (superficial skin layer).

varices Abnormally enlarged and convoluted veins, prone to bleeding, seen in the lower esophagus and stomach in association with portal hypertension.

vasculitis A group of diseases in which the primary pathology is inflammation of the blood vessels. Each of these diseases is differentiated by the characteristic distributions of blood vessel and organ involvement, and laboratory test abnormalities. Underlying immune system abnormality is a common feature.

Abbreviations

ALP	alkaline phosphatase
ALT	alanine transaminase
anti-HCV	hepatitis C Antibody
AST	aspartate transaminase
Bili	bilirubin
cccDNA	covalently closed circular DNA
DNA	deoxyribonucleic acid
EIA	enzyme immunosorbant assays
EMC	Essential mixed cryoglobulinemia
HAV	hepatitis A virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBIg	hepatitis B immune globulin
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis Delta virus
HEV	hepatitis E virus
HVR	hypervariable region
IFN	Interferon
Ig	immunoglobulin
IgM anti-HAV	hepatitis A IgM antibody
IgM anti-HBc	hepatitis B core IgM antibody
IL-12	interleukin-12
IM	intramuscularly
INR	international normalised ratio
IR	insulin resistance
IVDU	intravenous drug user
LCMV	lymphocytic choriomeningitis virus
LT	liver transplantation
MHC	major histocompatibility complex
NAFLD	nonalcoholic fatty liver disease
NANB	Non-A, non-B
NASH	nonalcoholic steatohepatitis
NHL	non-Hodgkin's lymphoma
NRTI	nucleoside reverse transcriptase inhibitor
ORF	open reading frame
RBV	ribavirin
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
SENV	SEN virus
SVR	Sustained viral response
TNF	tumor necrosis factor
TTV	Torque teno virus

Defining Statement

We discuss the pathogenesis of acute and chronic viral hepatitis, and the structure, replication, epidemiology, and clinical features of each hepatitis virus (A, B, C, D, and E). Current therapies for chronic HBV and HCV, vaccination against HAV, HBV, HEV, and newly identified hepatotropic viruses will also be covered.

Introduction

Hepatitis is a nonspecific term meaning inflammation of the liver (from the Greek *hepar* for liver + *itis* for inflammation) and does not necessarily imply a viral etiology. Many viruses can cause a systemic infection that may involve the liver with an acute hepatitis (e.g., cytomegalovirus, Epstein–Barr virus, and yellow fever virus). Hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis delta virus (HDV), and hepatitis E virus (HEV) are the five hepatotropic viruses responsible for most cases of acute viral hepatitis. Three of these, HBV, HDV, and HCV, also cause chronic hepatitis, with the risk of progression to cirrhosis, and hepatocellular carcinoma (HCC) (**Table 1**).

Before the identification of individual viruses, acute hepatitis was classified by British hepatologist F.O. MacCallum in 1947 as either infectious (transmitted via the fecal–oral route from person to person) or serum (acquired from the transfusion of blood and blood products). With the introduction of diagnostic tests in the 1960s and 1970s, HBV and HAV were identified as the major cause of serum and infectious hepatitis, respectively. In 1977, HDV was identified as a defective ribonucleic acid (RNA) virus that is dependent on HBV to replicate. However, not all individuals with acute infectious or serum hepatitis tested positive for HAV or HBV and it was strongly suspected there were additional causative viral agents. Non-A, non-B (NANB) hepatitis was characterized epidemiologically as either parenterally or enterally transmitted until two additional viruses were discovered in the 1980s: HCV and HEV. HCV was identified in 1989 as the major cause of parenterally transmitted NANB, and HEV in 1983 as the major cause of enterically transmitted NANB hepatitis. Some patients continue to have typical signs and symptoms of acute viral hepatitis, without serologic evidence of

Table 1 The major hepatitis viruses

	Hepatitis A	*Hepatitis B*	*Hepatitis C*	*Hepatitis D*	*Hepatitis E*
Family	Picornovirus	Hepadnae	Flavi virus	Unclassified	Hepeviridae
Genus	*Hepatovirus*	*Orthohepadna*	*Hepacivirus*	*Deltavirus*	*Hepatitis E-like virus*
Genome	RNA	DNA	RNA	RNA	RNA
Main transmission routes	Fecal–oral	Parenteral/ sexual	Parenteral	Parenteral	Fecal–oral
Materno-fetal transmission	No	High risk	Low risk	No	Possible
Chronicity	No	Yes	Yes	Yes	No
Commercially available vaccine	Yes	Yes	No	Prevented by hepatitis B vaccination	No

infection with any of the currently identified hepatitis viruses (non-ABCDE hepatitis).

HAV and HEV are transmitted via the fecal–oral route, whereas HBV, HDV, and HCV are spread predominantly through parenteral exposure; sexual contact and vertical transfer from mother to infant are generally significant routes of transmission only for HBV. Until recently, sexual transmission of HCV was not considered a significant route of infection; however, there is now accumulating evidence that high-risk sexual activity is a potential HCV risk factor for men who have sex with men.

There is wide geographical variation in the prevalence of these agents, with HAV and HEV endemic in developing countries reflecting an absence of safe, clean water supplies. HBV is highly endemic in sub-Saharan Africa and Asia, and it is estimated that about 5% of the world's population are chronically infected.

Common symptoms of acute hepatitis include fever, malaise, and anorexia (decreased appetite) in the 'prodromal' period, followed by the onset of dark-colored urine, pale stools, and jaundice. Although the majority will recover completely, a small proportion may develop severe fulminant hepatitis that carries a high mortality (50%) unless the individual undergoes liver transplantation (LT). Fulminant liver failure due to acute viral hepatitis is the most common emergency indication for LT. Chronic HBV and HCV infections are usually asymptomatic, unless there has been progression to cirrhosis when individuals can present with complications such as ascites and bleeding esophageal varices. Chronic HCV-related end-stage liver disease is the leading indication for LT in the Western world.

Histopathology of the Liver

Microscopically, the smallest functional anatomical unit of the liver is the 'lobule' (**Figure 1**). Each lobule consists of a branch of the hepatic vein (terminal hepatic venule) from which 'plates' of hepatocytes radiate toward a number of peripheral 'portal tracts', each tract composed of a biliary ductule, terminal hepatic arteriole, and terminal portal venule (the portal triad). In acute viral hepatitis, lobular rather than portal abnormalities dominate, whereas in chronic hepatitis changes are observed in the portal and periportal areas. Histological examination of the liver is not generally required in the setting of acute infection. In chronic viral hepatitis, liver histology is useful in assessing disease severity.

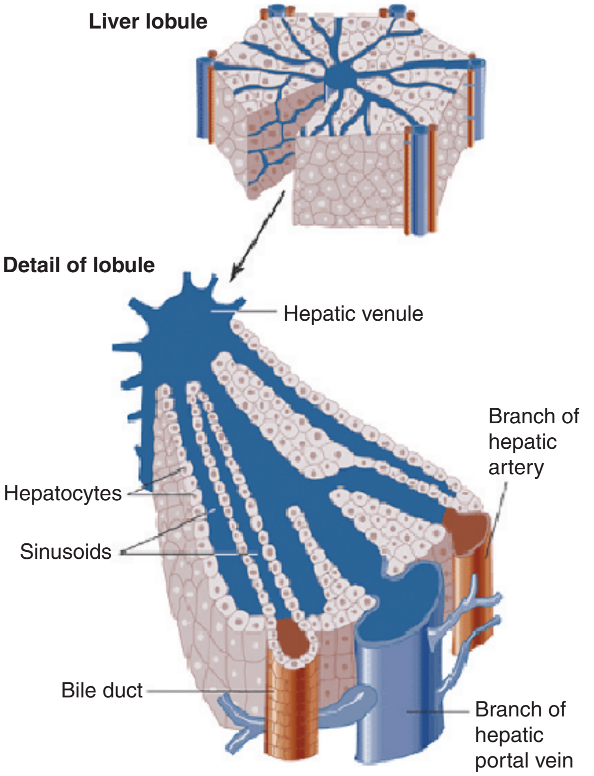


Figure 1 The structure of the liver's functional units, or lobules. Blood enters the lobules through branches of the portal vein and hepatic artery, then flows through small channels called sinusoids that are lined with hepatocytes Reproduced from Cunningham CC and Van Horn CG (2003) Energy availability and alcohol-related liver pathology. *Alcohol Research and Health* 27(4): 281–299, with permission.

Acute Viral Hepatitis

The etiology of an acute hepatitis caused by any of the five hepatitis viruses cannot be distinguished solely by clinical or biochemical features, and requires serological testing. Common prodromal symptoms include malaise and anorexia (loss of appetite), followed by dark-colored urine, pale stools, and icterus (jaundice). Although most cases of acute viral hepatitis resolve uneventfully, some patients with acute HBV and HCV become chronically infected. A minority of patients with acute HAV, HBV, and HDV (and also with acute HCV but far less likely) will develop fulminant hepatitis with acute liver failure. Affected individuals can also be asymptomatic, identified only after the infection has resolved, by positive serology performed incidentally (e.g., before blood donation).

Histology of Acute Viral Hepatitis

The histological picture is similar in acute hepatitis caused by any of the major hepatitis viruses. Characteristic findings include lobular disarray, diffuse hepatocyte injury, ballooning, eosinophilic degeneration, and necrosis, together with a predominantly mononuclear inflammatory response in the parenchyma and portal tracts. The inflammatory infiltrates consist mainly of T-cell lymphocytes, reflecting the role of cellular immunity in the pathogenesis of hepatitis. Although 'interface hepatitis,' formerly referred to as 'piecemeal necrosis' (the destruction of hepatocytes at an interface between liver parenchyma and connective tissue), is the defining feature of chronic hepatitis, it is also seen in acute hepatitis, particularly with HBV. Cholestasis may also be observed. As the reticular framework of the liver is usually preserved in acute hepatitis, hepatocyte regeneration and complete restoration of the liver normally occur after resolution of infection.

Typical features of acute HAV infection include hepatocellular injury and necrosis that predominates around the portal tracts, and a portal and periportal inflammatory infiltrate that contains abundant plasma cells. In adult-acquired acute HBV, histological findings are characterized by severe centrilobular necrosis and inflammation. Portal lymphoid aggregates and bile duct lesions of the Poulsen–Christoffersen type are characteristic of acute HCV, occurring rarely in other types of acute viral hepatitis (**Figure 2**).

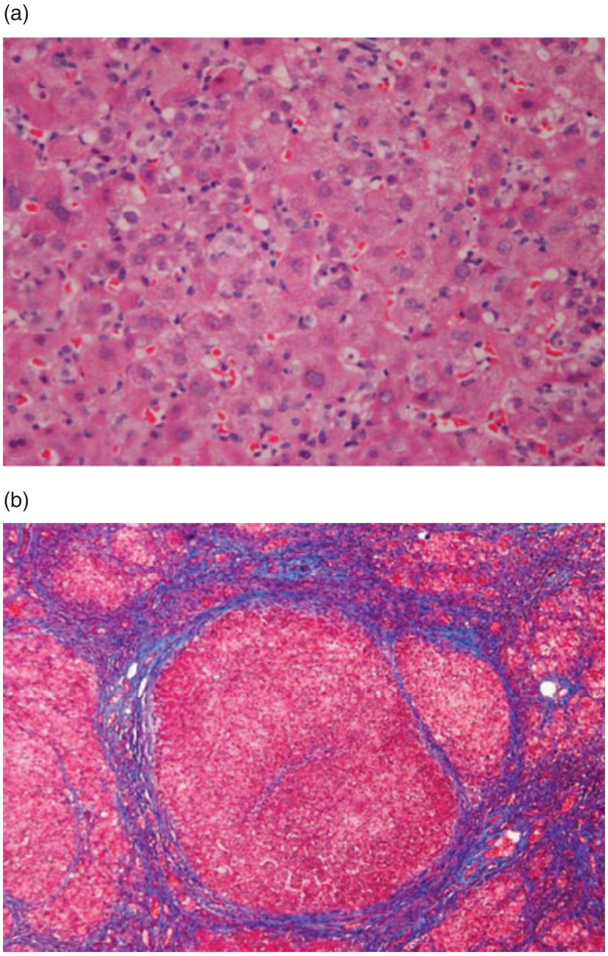


Figure 2 Histological features of (a) acute recurrent hepatitis C in a liver allograft showing diffuse necro-inflammatory changes in the parenchyma with acidophilic bodies, inflammation, and ballooning degeneration of hepatocytes; and (b) chronic hepatitis C with nodular cirrhosis. Courtesy of Dr. S. Thung, Mount Sinai School of Medicine, New York.

Acute Viral Hepatitis – Pathogenesis

Most hepatitis viruses are noncytopathic, liver damage in the acute as well as the chronic stages reflects the host immune response, largely controlled by CD4 T-helper lymphocytes. These immune responses are directed at viral- or self-antigens expressed on the surface of infected hepatocytes via the major histocompatibility complex (MHC). In acute HBV, the antiviral cytotoxic T-lymphocyte response is directed against multiple epitopes within the HBV core, polymerase, and envelope proteins. The mechanism of cytotoxic T-lymphocyte destruction of HBV-infected hepatocytes has been investigated in a mouse model, with the number of infected hepatocytes killed by direct interaction between cytotoxic T lymphocytes and their targets insufficient to explain the extent of damage observed in acute hepatitis. It has therefore been postulated that much of the injury is due to secondary antigen-non-specific inflammatory responses induced by the response of the cytotoxic lymphocytes (e.g., due to release of tumor necrosis factor (TNF), free radicals, and proteases), and also possibly due to the involvement of other immune cells such as natural killer T cells.

It is believed that the immune response to one or more viral proteins is responsible for both viral clearance and liver injury during infection.

Acute Viral Hepatitis – Clinical Features

Parenterally transmitted hepatitis viruses tend to have longer incubation periods than those transmitted via the enteric route. An incubation period of between 40 and 160 days is observed in HBV and HDV infection, 15–160 days with HCV, 14–60 days for HEV, and 10–50 days for HAV.

In symptomatic individuals, after a typical viral prodrome, symptoms of anorexia, right upper abdominal discomfort, with dark urine and pale stools, precede the onset of icterus by several days. On examination, the liver is usually enlarged/palpable and mildly tender, although marked hepatomegaly or splenomegaly is uncommon. Once the patient is icteric, fever resolves and constitutional symptoms generally improve. The duration of jaundice can vary from a few days to several weeks. In the majority of cases, acute viral hepatitis is a self-limited disease with patients recovering completely by 2–8 weeks after the onset of jaundice. Occasionally a patient may experience 'biphasic hepatitis', where initial clinical improvement is followed by a relapse of signs and symptoms. This is most often seen in HAV infection; if this situation occurs in a patient with acute HBV, the possibility of acute HDV should be considered. Although wide fluctuations in alanine transaminase (ALT) occurring over weeks to months can be seen in acute HCV, a true 'biphasic' course is uncommon. Approximately 10% of subjects with acute HBV initially develop a serum-sickness-like syndrome (characterized by skin rash, angioedema, and arthritis) due to circulating immune complexes of viral particles and antibody with complement activation (see 'Extrahepatic manifestations of viral hepatitis').

Fulminant Acute Viral Hepatitis

The majority of cases of fulminant hepatitis (>50%) are due to acute HBV (with and without HDV superinfection). Fulminant disease is only seen in a small percentage of patients with HAV (this is more likely if the disease is acquired by older adults), and very rarely associated with acute HCV. Between 1 and 2% of cases of HEV can also become fulminant, particularly when acquired by females in the later stages of pregnancy when the percentage increases to over 20%. Individuals with underlying chronic liver disease, such as HCV-related cirrhosis, who are infected acutely with HBV, HAV, or even HEV, are at increased risk for a fulminant course. In patients with chronic HBV, hepatitis flares and rarely fulminant hepatitis can accompany changes in immunological response to the virus, for example, reversion from nonreplicative to replicative infection (hepatitis B e antigen (HBeAg-to-anti-HBe seroconversion), and can also be seen in individuals with previously quiescent chronic HBV undergoing immunosuppressive or cancer chemotherapy. Although only a small percentage of patients with acute viral hepatitis develop fulminant hepatitis, altered mental status in an icteric patient should prompt referral to a transplant center because of the high mortality rate.

Acute Viral Hepatitis – Laboratory Features and Diagnosis

Acute viral hepatitis can usually be differentiated from other causes of acute jaundice by the marked elevations of two liver enzymes measured in serum, ALT, and aspartate transaminase (AST). ALT is typically higher than AST, but absolute levels correlate poorly with clinical severity. Serum transaminases begin to increase during the prodromal period, preceding the onset of jaundice, and can reach a peak level of >1000°IU°l^{-1} (normal range 10–40 IU l^{-1}). The serum bilirubin (Bili) is mainly conjugated and reflects the severity of the hepatitis; however, large elevations in transaminases can be seen without significant elevations in Bili, particularly in acute HCV. Alkaline phosphatase (ALP) is usually only mildly to moderately elevated, marked elevation suggests extrahepatic cholestasis and should prompt imaging, for example, with ultrasound. Occasionally, there may be a cholestatic phase (most commonly seen with HAV infection) when Bili and ALP levels remain elevated in the face of decreasing transaminases, with the patient exhibiting symptoms of severe pruritis (itching) and jaundice. Assessment of the synthetic function of the liver with albumin and coagulation tests, that is, prothrombin time and International normalized ratio (INR), provides the most sensitive measure of liver injury. Increasing prolongation of the INR implies possible hepatocellular failure and evolution to fulminant hepatitis.

The most useful initial serological tests are hepatitis B surface antigen (HBsAg), hepatitis A IgM antibody (IgM anti-HAV), hepatitis B core IgM antibody (IgM anti-HBc), and hepatitis C antibody (anti-HCV) (**Figure 3**). Acute HAV is confirmed by IgM anti-HAV. Acute HBV is indicated by the presence of HBsAg with IgM anti-HBc. Unless there is documented anti-HCV seroconversion after a discrete HCV exposure, such as a needlestick injury, acute HCV can be difficult to diagnose conclusively. Viral replication can be confirmed by the detection of HCV RNA in serum, but does not distinguish acute from chronic infection. If HEV infection is suspected, the diagnosis can be confirmed with serology. Both IgM and IgG anti-HEV can be seen in acute infection.

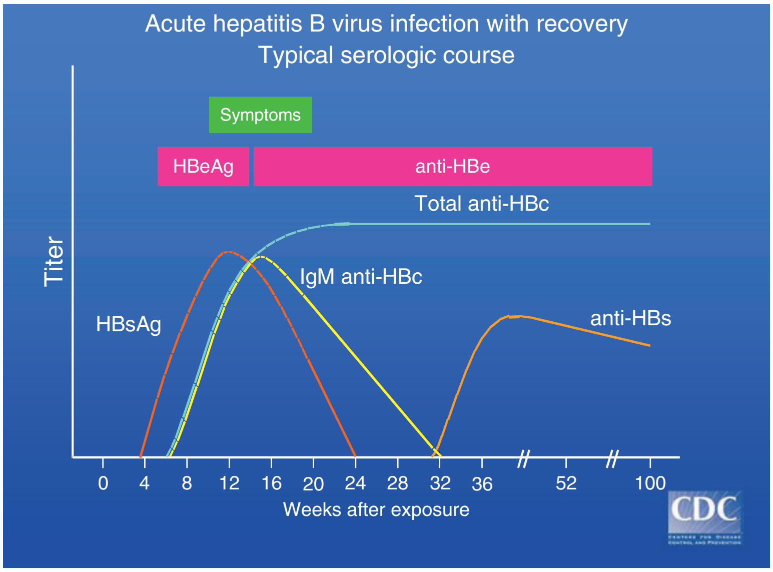


Figure 3 Graphical representation of evolution of serological markers in acute hepatitis B infection. Reproduced from Center for Disease Control and Prevention, USA, with permission.

Acute Viral Hepatitis – Treatment

Most cases of acute viral hepatitis resolve spontaneously and require no specific treatment other than supportive care. Small series, however, suggest that treatment of acute HBV may abort its course and prevent chronicity. Owing to the high risk of progression to chronicity in acute HCV, with up to 75% of those infected failing to clear the virus, there is valid reason to advocate antiviral therapy for those individuals who are persistently viremic 4–6 months after exposure. Sustained viral response (SVR) rates of over 90% have been reported using interferon (IFN) monotherapy.

Extrahepatic Manifestations of Viral Hepatitis

Extrahepatic manifestations of acute and chronic viral hepatitis are most frequently seen with acute or chronic HBV with immune complex-mediated tissue damage. A 'serum sickness'-like syndrome sometimes seen in acute HBV is thought to be due to the deposition of circulating immune complexes of HBsAg-anti HBs in blood vessel walls, leading to the activation of the complement system and depressed serum complement levels.

Other types of immune-complex disease may be seen in chronic HBV, including membranous glomerulonephritis and, rarely, polyarteritis nodosa. Deposition of HBsAg, immunoglobulin (Ig), and C3 has been described in the glomerular basement membrane; membranoproliferative glomerulonephritis has also been reported with chronic HCV. HBV is implicated in up to 20% of cases of childhood membranous nephropathy.

Essential mixed cryoglobulinemia (EMC), initially associated with hepatitis B, has subsequently been shown to be more frequently associated with chronic HCV (>90% cases). Cryoglobulinaemia is characterized by the presence of Igs in the blood (cryoglobulins), which are precipitated into the microvasculature at low temperatures, and then redissolve at temperatures of 37 °C. In EMC, cryoprecipitable immune complexes of more than one Ig class are found in the serum; these immune complexes contain HCV RNA. Clinical features of EMC include arthritis, cutaneous vasculitis (palpable purpura), and occasionally glomerulonephritis. EMC can be associated with overt lymphoproliferative disorders of B-cell lineage. HCV is a factor in the pathogenesis of at least a proportion of patients with non-Hodgkin's lymphoma (NHL), with some cases of HCV-associated NHL being highly responsive to antiviral therapy.

A variety of other extrahepatic diseases such as Sjögren's syndrome, autoimmune thyroiditis, porphyria cutanea tarda, and lichen planus has been described in chronic HCV. The mechanism by which HCV causes these extrahepatic diseases is still unclear. Antiviral therapy with IFN, with or without ribavirin (RBV), leads to remission or resolution in many cases.

Chronic Viral Hepatitis

Acute infections with HAV and HEV infections do not progress to chronicity and therefore there is no risk of long-term liver damage. Acute infection with HBV, HDV, and HCV, however, can become chronic, with persisting viral replication 6 months after initial infection. Chronic infection with HBV occurs most commonly in individuals who acquire the virus perinatally or during early childhood when the immune response is muted, whereas exposure in later life through sexual contact or IV drug use leads to chronic infection in less than 5% of cases. Both viral and host factors are thought to contribute to the high rate of chronicity with HCV infection (approximately 80%), which unlike HBV is not related to age of exposure. Neither HBV nor HCV is predominantly cytopathic; hence, inflammation and injury in chronic infection are probably immune mediated. Chronic HBV and HCV infections are often asymptomatic, patients may complain of vague right upper abdominal discomfort, and nonspecific symptoms such as chronic fatigue and malaise. The natural history of chronic HBV is complex, but recent data suggest that progression to cirrhosis and HCC is correlated with serum HBV DNA levels and HBV genotype. In HCV, up to one-third of chronically infected individuals, if untreated, will progress over a variable time period (usually 20–30 years) to cirrhosis; however, in contrast to HBV, viral factors such as HCV RNA level and genotype do not appear to influence disease progression. The rate of fibrosis progression in chronic HBV and HCV infections is also dependent on host variables, for example, age, immunogenetics, and alcohol consumption. Once cirrhosis develops, additional manifestations include portal hypertension with bleeding esophageal varices and ascites, and impairment of hepatocellular function. The risk of HCC is also increased in cirrhosis, whatever the underlying cause, although the risk is particularly high if the individual has chronic HBV (relative risk increased by >200). However, unique among most causes of liver disease, HCC can occur in chronic HBV infections in the absence of cirrhosis.

LT in Chronic Viral Hepatitis

There is currently no treatment other than LT to offer individuals with chronic HBV or HCV and advanced liver disease with complications such as portal hypertension and ascites. However, recurrence of the original infection after LT is an important cause of morbidity and mortality. The early results of LT for HBV were disappointing due to the high rates of graft loss from severe cholestatic recurrent HBV infection. LT became a viable option once hepatitis B immune globulin (HBIg) had been shown to reduce recurrence rates to below 10%. More recently, the antiviral drugs lamivudine and adefovir have been used, with and without HBIg, to prevent recurrence. Unfortunately, there is currently no therapy available to prevent recurrent infection with HCV, and reinfection after LT is almost universal. Recurrent HCV is variable in severity, but some individuals progress to cirrhosis within 3–5 years of transplant. In a large US database (>11 000 patients), 5-year survival rates after LT were reduced in HCV-positive compared with HCV-uninfected individuals (69.9 vs. 76.6%).

Histology of Chronic Viral Hepatitis

HBsAg-positive ‘ground glass’ hepatocytes are characteristic of chronic and not acute HBV infection. Identification of cytoplasmic HBsAg is possible with special stains (Shikata orcein or Victoria blue stains) or immunohistochemically. Accumulation of hepatitis B core antigen (HBcAg) in hepatocyte nuclei produces an appearance known as ‘sanded’ nuclei. HBcAg is best identified using immunohistochemical staining. Coinfection with HBV and HDV produces the same histological picture as with HBV alone, but often with a more severe degree of necro-inflammation. Hepatitis D antigen can be visualized using immunohistochemical staining.

Characteristic histological features of chronic HCV infection include dense portal lymphocytic infiltrates, often with lymphoid aggregates and sometimes with follicle formation accompanied by varying degrees of interface hepatitis. Mild degrees of biliary epithelium damage (the ‘Poulsen lesion’) can also be seen. There is currently no commercial immunohistochemical stain for HCV. Hepatic steatosis can be observed in up to 70% of liver biopsies from individuals with chronic HCV (74% in genotype 3 vs. 50% in non-3 genotype) as compared with up to 18% of patients with chronic HBV infection. The high prevalence is due to a combination of the direct steatogenic effect of HCV (particularly genotype 3) and the prevalence of metabolic risk factors in the HCV population. Steatohepatitis has been shown to enhance fibrosis progression in chronic HCV.

Chronic Viral Hepatitis – Pathogenesis

After acute infection with HBV or HCV, impairment of hepatitis virus-specific T-cell responses can lead to failure to clear the virus, with the establishment of chronic infection. Individuals who spontaneously clear HBV and HCV maintain durable virus-specific CD4+ and CD8+ T-cell responses that can be easily detected in the blood for decades, whereas those who progress to chronic viral hepatitis typically display narrowly focused and weak HBV- and HCV-specific T-cell responses. Virus-specific T cells in these subjects show reduced proliferation and production of cytokines, and there is reduced cytotoxicity of CD8+ T cells. The gradual loss of T-cell function is termed ‘exhaustion,’ and is thought to be secondary to the frequent exposure to a high viral and antigenic load, for example, in chronic HBV, large quantities of HBeAg are secreted into the blood and may be the cause of neonatal T-cell tolerance and in altering the reactivity of HBe-specific CD8+ T cells. In HCV, it has been demonstrated that other antigen-specific mechanisms are involved in the downregulation of cellular immune responses, even at low antigen concentrations; for example, recombinant HCV core protein has been shown to downregulate interleukin-12 (IL-12) production by macrophages *in vitro*.

Reduction of effector T-cell function occurs in the following order: IL-2 production being affected first, then cytotoxicity and the production of TNF-α, with IFN-γ production usually preserved until last. In addition, the functional potential of CD8+ T cells can be negatively impacted if help from CD4+ cells is reduced or unavailable. Finally, viral mutations (which are more frequent in HCV than in HBV infection) also contribute to CD8+ T-cell impairment by affecting the intracellular processing of T-cell epitopes, their binding to major histocompatability molecules, and the stimulation of T-cell receptors. These mutations also affect recognition by specific antibodies, so that all arms of the adaptive immune response are downregulated.

A small number (<2% per annum) of chronically infected HBV patients spontaneously clear HBsAg and develop neutralizing antibodies. In these individuals, HBV-specific T-cell responses become detectable in the blood just before seroconversion. This implies that latent, immune-mediated clearance mechanisms can become spontaneously activated in chronically infected subjects. It is not known whether such immune responses can be induced therapeutically by vaccination and or antiviral therapy in patients with chronic HBV. Studies in animal models of related chronic viral infections (e.g., the mouse model of chronic infection with the1 hepatotropic lymphocytic choriomeningitis virus, LCMV) suggested that proliferative CD8+ T-cell responses can be partially restored if viral load was reduced with antivirals before vaccination. However, results of studies of HBV peptide and protein vaccination in adults and children with HBV have been disappointing, and although therapy with nucleoside analogues results in a transient recovery of HBV-specific CD4+ and CD8+ T-cell function in peripheral blood, this does not persist for greater than 6 months. Antiviral therapy in chronic HCV is not associated with even a transient increase of HCV-specific T-cell responses. Extrapolating on data obtained from the LCMV-infected mouse model, it would appear that virus-specific CD8+ T cells develop an intrinsic defect during chronic infection that prevents a vigorous proliferative response to vaccination even if the virus is removed.

HAV

HAV is a nonenveloped positive-strand RNA virus transmitted via the fecal–oral route. Although globally distributed, risk of infection decreases as levels of hygiene and sanitation improve, and an effective preventative vaccine is available.

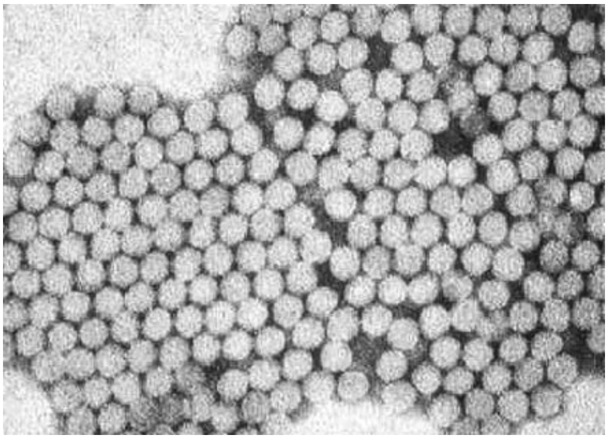


Figure 4 Electron micrograph of hepatitis A virus particles. Reproduced from Center for Disease Control and Prevention, USA, with permission.

Virology

First visualized by immune electromicroscopy in 1973, HAV has been classified within a separate genus of the *Picornaviridae* family, the genus *Hepatovirus* (**Figure 4**). The virus is nonenveloped, with a diameter of 27–32 nm, with a linear RNA genome The 3′-end terminates with a poly (A) tail of 40–80 nucleotides The genome contains a single, large open reading frame (ORF), which codes for a large polyprotein and which is subsequently cleaved by a viral protease, forming three capsid proteins and several nonstructural proteins. The capsid is composed of 60 copies of each of three major structural proteins, VP1, VP2, and VP3.

Once the virus enters the hepatocyte, there is loss of the protein coat releasing the positive-sense RNA strand into the cytoplasm. Genomic replication takes place by a mechanism involving an RNA-dependent RNA polymerase. HAV then enters the biliary tract and exits the body via the feces. Several viral genotypes have been identified, but with only one serotype, with one immunodominant neutralization site, a property that facilitated the development of an effective vaccine.

Epidemiology

HAV is transmitted via the fecal–oral route, by ingestion of contaminated food or water or direct person-to-person transmission; it can occur either sporadically or in large outbreaks. The highest prevalence of this infection occurs in countries with lack of access to clean water supplies. In endemic areas, infections occur early in life, such that most children by the age of 9 are seropositive for HAV. In contrast, HAV is mainly a disease of adults in most industrialized nations, who are much more likely to have symptomatic infection. Despite high seroprevalence rates in highly endemic populations, HAV perpetuates in these regions due to its ability to survive in the environment for prolonged periods, and, once ingested, to withstand the acid environment of the stomach. Food and water can become contaminated with HAV by the use of human feces as fertilizer, and the pollution of water supplies by raw sewage. Consumption of raw or undercooked shellfish is associated with infection as certain mollusks can concentrate HAV from polluted waters. Parenteral transmission of HAV is possible, with cases reported in intravenous drug users, hemophiliacs, and rarely via blood transfusion. Sexual oro-anal contact can also result in transmission.

Clinical Features

The incubation period is from 10 days up to 7 weeks, during which time high levels of HAV are present in liver tissue, bile, and feces (virus can also be detected in blood in lesser amounts); the infected individual is generally asymptomatic and highly infectious. After the 4th or 5th week of infection there is an immune response accompanied by the first symptoms (the prodromal or preicteric period), which may include fatigue, fever, anorexia, nausea and vomiting, diarrhea, and right-sided abdominal pain. During this period, ALT and AST are increasingly elevated. Generally, within 10 days of the onset of symptoms the patient enters the icteric phase of infection, with resolution of fever and marked decrease in viremia, although virus is still shed in the feces for another 1–2 weeks. Extrahepatic symptoms are uncommon, although urticaria can occur. Jaundice usually resolves over 1–2 weeks, and a complete recovery can take up to 6 months. The neutralizing antibodies produced in an infected individual can be used therapeutically to prevent infection in an exposed individual (hyperimmune or γ-globulin).

Diagnosis

Routine diagnosis is usually made by the detection of IgM anti-HAV. Experimentally, infection with HAV can be confirmed by the detection of virus in the blood or feces. Antibodies to HAV of the IgM class can be detected approximately 3 weeks after exposure, and these increase in titer over the next 4–6 weeks before disappearing from the blood. Shortly after the appearance of IgM anti-HAV antibodies, IgG anti-HAV antibodies can be detected; these persist for years after resolution of acute infection, conferring lasting immunity. As the presence of IgG anti-HAV in the serum can indicate either prior or current infection, it is therefore important to request both IgM anti-HAV and IgG anti-HAV to establish the diagnosis of acute HAV.

Vaccines

There are four inactivated commercial vaccines currently available for the prevention of HAV. These are safe, highly effective, and provide long-term protection (for at least 20 years) against infection; however, a booster vaccination after 10 years is recommended. Although generally used preexposure, mass postexposure vaccination has been successfully utilized to contain the spread of HAV in established outbreaks. Dosing schedules vary, but generally two doses of vaccine are administered intramuscularly (IM) 6 months apart. This will produce protective levels of antibody within 1 month of the first dose in almost 100% of those vaccinated. These vaccines can be coadministered with others, including live attenuated vaccines, without affecting immunogenicity.

Vaccination strategies differ between areas of high and low endemicity. In most developing countries, HAV is acquired in early childhood and tends to be asymptomatic, and therefore HAV is not a major public health issue requiring universal immunization. As nations develop public sanitation, the age at which individuals will become infected is delayed until adulthood, and subsequently the risk of symptomatic illness is much higher. In such countries, groups at high risk of infection due to lifestyle or occupation, and travelers to areas of high endemicity, are targeted for vaccination. One study from Ireland showed that if HAV immunity in a population is greater than 45%, screening followed by vaccination of nonimmune individuals is the most cost-effective strategy; if less than 45%, vaccination without prior screening should be used.

Ig

This is more often used in the setting of postexposure prophylaxis or in situations where immediate protection is required. Nonimmune individuals should be given $0.02\ \mathrm{ml\,kg^{-1}}$ of Ig within 2 weeks of exposure, in which case it will either prevent or attenuate the severity of the infection. Ig can safely be given to children, pregnant and lactating women, and the immunocompromised, and a single dose of 100 IU given IM will provide protection for up to 6 months. Ig should not be coadministered with live attenuated vaccines as this may interfere with the immune response to these vaccines, but can be given with HAV vaccine (at a different site) for maximal protection after an exposure.

HBV

Uniquely among DNA viruses, HBV replicates within infected hepatocytes through a process called reverse transcription. Infection with HBV can result in different clinical scenarios, ranging from fulminant hepatitis to asymptomatic chronic infection, cirrhosis, or HCC. Integration of HBV viral DNA into the hepatocyte nuclear DNA is a key event in hepatocarcinogenesis. HCC resulting from chronic HBV is one of the most common malignancies worldwide.

Virology

HBV is a member of the family Hepadnaviridae, with a double-stranded circular DNA genome that replicates through an RNA intermediate. Although HBV infects only human and higher primates, related viruses are common in rodent (e.g., woodchuck) and bird species (e.g., duck). Hepadnaviridae preferentially infect liver cells, with trace amounts of HBV DNA found in kidney, pancreas, and mononuclear cells without causing disease at these sites. HBV DNA does not integrate into host cellular DNA as part of the viral replication cycle, but may persist in the host cell for many years even after clinical resolution of infection. During this time, HBV DNA may be integrated into cellular DNA and it is thought that integration plays a role in the subsequent development of HCC.

Each HBV particle consists of a 3.2-kb double-stranded circular DNA genome and DNA polymerase enclosed by a viral envelope, containing three related surface proteins, and an icosahedral nucleocapsid of approximately 30 nm diameter.

HBV replication proceeds through a unique and complex mechanism utilizing an RNA intermediate, with viral RNA transcripts being generated from a covalently closed circular DNA (cccDNA) transcriptional template found in the nucleus of the host cell in the form of a viral mini chromosome. The genome encodes four ORFs, which when translated produce the precore and core, polymerase, small, medium, and large envelope proteins, and the transcriptional regulator protein X. The core gene can also produce a small-molecular-weight protein called HBeAg by an alternate start codon and post-translational modification. The HBV polymerase has a number of functions in the viral life cycle, including the initiation of minus-strand DNA synthesis (priming), the synthesis of DNA using either RNA or DNA templates (polymerase activity), the degradation of the RNA strand of RNA–DNA hybrids (nuclease activity), and the packaging of the RNA pregenome into nucleocapsids.

There are eight HBV genotypes (A–H) so designated on the basis of a genomic variation of at least 8%. Coinfection with different genotypes has been reported. Genotype A is the most widely distributed, being frequent in northwest Europe, sub-Saharan Africa, India, and North, Central, and South America; B and C are common in Southeast Asia and Oceania, and D is prevalent around the Mediterranean, Central Asia, and South America. Genotype E is found only in West Africa, F is limited to Central and South America but is also found in Native Americans, G has been described in the United States and France, and H in Central America.

There is accumulating evidence that HBV genotypes may influence HBeAg seroconversion rates, mutational patterns in precore and core promoter regions, and the clinical severity of liver disease. For example, infection with genotype A HBV may be more likely to become chronic, but causes milder liver disease than other genotypes; it also has a better response to treatment with IFN. In contrast, genotype C HBV appears to be associated with a more aggressive clinical course and faster progression to cirrhosis. The predominant mutation in the precore region of the HBV genome, which completely aborts the production of HBeAg, is rarely seen with HBV genotypes A and F, but is found mainly in genotypes B–D.

The lifecycle of HBV begins with viral attachment and entry into the hepatocyte followed by internalization. Once in the cytoplasm, viral uncoating occurs and HBV DNA is transported to the nucleus where it is converted to cccDNA, a stable template for transcription of both subgenomic messenger RNA (for translation into viral proteins) and pregenomic RNA (for reverse transcription into genomic DNA). HBV cccDNA is responsible for viral persistence. After binding of the HBV polymerase and core Ag to the pregenomic RNA, encapsidation occurs. Synthesis of the negative DNA strand by reverse transcription and partial synthesis of the positive strand is performed by HBV polymerase within the nucleocapsid, which is then either transferred to the endoplasmic reticulum where it assembles with HBsAg molecules to form the virion, or returns to the nucleus to amplify the cccDNA reservoir. The intact virion exits the cell by budding and vesicular transport. The surface proteins can also bud in the absence of capsids, forming both spherical and tubular (or 'filamentous') particles approximately 22 nm in diameter consisting of HBsAg and host-cell-derived lipids (**Figure 5**). These particles are also secreted into the blood, as they do not contain viral nucleic acid and they are not infectious, but are highly immunogenic.

The turnover of virions is high, with estimates on the order of 10^{11} virions produced per day – much higher than that for other DNA viruses, HCV, or HIV. The HBV DNA polymerase does not have proofreading or editing capacity, and this together with the high rate of virion production makes replication errors inevitable. It has been calculated that over the period of 1 day, an estimated 10^{14} nucleotides are replicated, with the potential for 10^7 base pairing errors.

The consequence of this high mutational rate is the formation of a large pool of 'quasispecies' with the virus, with the best replicative 'fitness' becoming the dominant species. Selection pressure, either from the host immune response or from antiviral therapy, can result in vaccine/Ig escape mutants and drug-resistant variants.

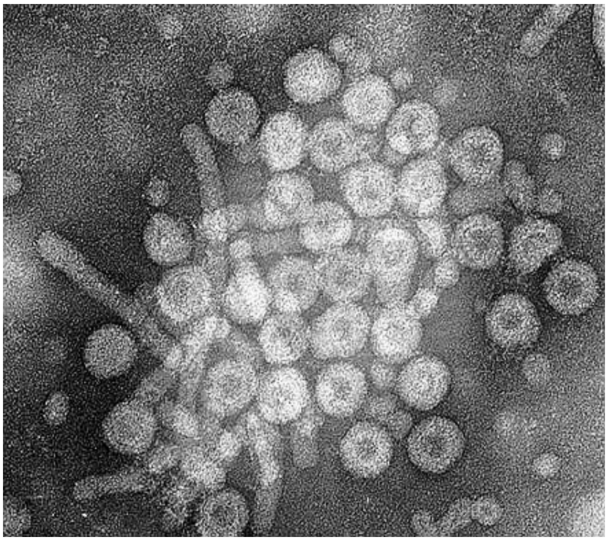


Figure 5 Electron micrograph of hepatitis B virus showing spherical, tubular, and double-shelled forms. Courtesy of Dr. Arie Zuckerman.

Mutations of the HBV envelope, precore, core, and polymerase regions occur. Several precore mutations have been identified in HBeAg-negative patients. The most common mutation observed is a G → A substitution at nucleotide 1896 (G1896A) in the precore region, creating a premature stop codon. Translation of the precore protein is prevented, which aborts the production of HBeAg.

Epidemiology

There are an estimated 350 million people in the world with chronic HBV, with prevalence varying widely between geographical areas. HBV is efficiently transmitted horizontally by intimate contact, by parenteral exposure as well as vertically from mother to infant. However, in about a one-third of adults with acute infection, no risk factors are identified, probably reflecting HBV's ability to survive outside the body for up to a week as well as its ubiquitous presence in body fluids. Vertical transmission occurs peripartum or in infancy rather than *in utero*. The risk of vertical transmission is increased if the mother is HBeAg positive with high serum HBV DNA.

Countries with low prevalence in the general population (i.e., carriage rates of HBsAg of less than 2%) include the United States, Canada, Northern, Western, and Central Europe, and Australia; however, within these countries higher prevalence rates are seen in high-risk populations, for example, in intravenous drug users (IVDUs) and in individuals with multiple sexual partners. Intermediate carriage rates (2–8%) are observed in Eastern Europe, the Mediterranean, Russia and the Russian Federation, Southwest Asia, and Central and South America. The highest prevalence of HBsAg positivity (8% and above) occurs in China, Southeast Asia, Alaska, and tropical Africa. The age at which most HBV transmission occurs varies according to the prevalence rate. The majority of individuals with chronic HBV in countries with high prevalence of HBV acquire infection vertically or horizontally during early childhood. In low-prevalence areas, the highest incidence of infection occurs in teenagers and young adults, reflecting sexual and parenteral transmission. Iatrogenic transmissions from contaminated blood transfusions, use of unsterile needles for injections, or vaccinations remain risk factors in the less-developed world.

Clinical Features

Acute infection

The incubation period for HBV infection ranges from 6 weeks to 6 months. Clinical presentation reflects host age and immune status of the host. Acute perinatal or childhood infection is generally asymptomatic. Children older than 5 years and adults are symptomatic in 33–50% of infections with typical signs and symptoms of acute hepatitis. Fulminant hepatic failure occurs in approximately 1% of patients with acute HBV. Most adults with acute HBV recover completely without sequelae, although in $\leq$5% infection persists into chronicity.

Chronic infection

The natural history of chronic HBV is dynamic, with several phases characterized by differing levels of serum ALT, HBeAg, and HBV DNA. Chronic HBV acquired in infancy or in early childhood often has a prodromal 'immune-tolerant phase,' which can last up to young adulthood, characterized by HBeAg positivity, high levels of serum HBV DNA, but with normal levels of ALT and minimal inflammation on liver biopsy. In the late teens or early adulthood, a transition occurs to a more active phase of 'immune clearance', during which time serum HBV DNA levels fall with increasing ALT levels and worsening liver inflammation (classic HBeAg-positive chronic HBV). This phase of active replication is usually asymptomatic, but can be prolonged, with ultimate progression to cirrhosis, although it ends in HBeAg-to-anti-HBe seroconversion at a rate of approximately 10–15% of individuals per annum. Increased immune responsiveness can be accompanied by an abrupt 'flare' in ALT levels, which may even mimic fulminant hepatitis. The third phase, 'low-replicative' or inactive carrier state, is characterized by detectable HBsAg and anti-HBe, the absence of HBeAg, and low ($<10^5$ copies per ml) levels of HBV DNA, with a reduction in necro-inflammation on biopsy. However, spontaneous reactivation, with an increase in markers of disease activity (HBV DNA and ALT) without the reappearance of HBeAg, occurs in up to a one-third of individuals (classic HBeAg-negative chronic hepatitis B) owing to mutations in the HBV genome, which abolish HBeAg production but allow a return of HBV replication. HBeAg-negative chronic HBV is characterized by fluctuating ALT levels and progressive liver injury and reflects a later stage of the disease.

Diagnosis of HBV infection

There are a number of laboratory tests that can be used to diagnose hepatitis B infection, and also to determine whether the individual is in the acute or chronic phase (**Table 2**).

Therapy of Chronic HBV infection

Goals of HBV therapy include suppression of viral replication with the prevention of progression of liver disease and the development of HCC. Recent large, prospective, cohort-based studies following a large Taiwanese population of HBsAg-positive patients for a median of 11 years have concluded that serum HBV DNA level is a key predictor of progression to cirrhosis and HCC, independent of ALT and HBeAg status. Increasingly elevated serum HBV DNA is being proposed as the major criteria to initiate antiviral therapy. A detailed discussion of treatment guidelines is outside the scope of this article, but generally noncirrhotic individuals with chronic HBV, elevated ALT, and HBV DNA levels of $\geq$20 000 IU ml^{-1} for eAg-positive disease and $\geq$2000 IU ml^{-1} in eAg-negative disease should be given treatment. If HBV DNA levels fit these criteria but ALT is normal, the patient should have regular monitoring of ALT and a liver biopsy should be considered, and treatment instituted if there is significant histological disease. Patients with chronic HBV and cirrhosis are candidates for therapy regardless of ALT and HBV DNA levels because of the risk of frank hepatic decompensation with a spontaneous flare in cirrhotics. A number of end points are used to assess treatment response, normalization of elevated ALT (biochemical), achieving undetectable HBV DNA in the serum using a sensitive assay, and/or loss of HBV surface and eAg (virological), and improvement in liver necro-inflammation and fibrosis (histological). IFN-α has been used to treat chronic HBV since the mid-1980s; more recently a number of oral antiviral drugs have also become available. Whereas IFN is contraindicated in individuals with decompensated cirrhosis, the oral agents have the advantage of being safe and well tolerated in this patient population. Currently six agents are licensed for the management of chronic HBV, they are: IFN-α 2b, pegylated IFN-α 2a, lamivudine, adefovir, and, more recently, entecavir and telbivudine. Efficacy of IFN therapy is strongly dependent on HBV genotype in eAg-positive disease, with best results seen in genotype A and B infections. Lamivudine, a nucleoside analogue reverse transcriptase inhibitor (NRTI), was originally developed for the treatment of HIV infection, but was found to also inhibit the replication of HBV and was subsequently licensed for use in chronic HBV in 1998. NRTI's interfere with viral replication by causing premature termination of the DNA chain; other recently licensed members of this drug class include entecavir and telbivudine. Adefovir, introduced in 2002, also works by DNA chain termination but is a nucleotide analogue. Although these drugs effectively suppress HBV replication, they have much less effect on clearing the 'reservoir' of cccDNA in hepatocytes. Viral relapse will usually occur once therapy is discontinued, unless the subject has eAg-positive chronic HBV and achieves seroconversion with the loss of eAg and production of anti-HBe. If seroconversion occurs, therapy can be discontinued 12 months after seroconversion with low chance of relapse. In patients with eAg-negative disease, it is impossible to recognize a treatment-induced seroconversion, and therefore therapy may need to be continued indefinitely.

Table 2 HBV serology and interpretation of diagnostic tests

Test	*Acute HBV*	*Past exposure (immunity)*	*Previous immunization*	*Chronic HBV Infection (HBeAg^{+})*	*Chronic Precore Mutant (HBeAg^{-})*
HBeAg	+	−	−	+	−
Anti-HBe	−	−/+	−	−	+
HBsAg	+	−	−	+	+
Anti-HB^{s}	−	+	+	−	−
Anti-HBc	+	+	−	+	+
IgM anti-HBc	+	−	−	−[a]	−
HBV DNA	+	−	−	+	+
ALT	Elevated	Normal	Normal	Normal or elevated	Normal or elevated

[a] May become detectable in severe reactivations of chronic HBV.

Adapted from Hepatitis B Management Algorithim, Hepatitis Resource Network, with permission.

The development of resistance to oral agents with prolonged use is a cause for concern, recalling the situation seen in the early days of antiretroviral therapy for HIV. There is now a commercially available test that detects the key genotypic mutations in HBV polymerase associated with antiviral therapy with some of the most frequently used oral drugs (lamivudine, adefovir, and entecavir). The most efficacious way to use these drugs to minimize the development of resistance is currently under evaluation, but may involve using two or more drugs in combination.

Vaccines

Several preventative HBV vaccines are currently available; these rely on the use of one of the viral envelope proteins (HBsAg) and are now made using recombinant technology. HBV vaccines are available in monovalent formulations and in combination, for example, with HAV vaccine. Two recombinant vaccines are available; both are safe, even during pregnancy. Three 1.0 ml IM injections are given into the deltoid or anterolateral thigh, at time 0, 1 month, and 6 months, although there is flexibility and an accelerated schedule can be used. Children are given lower doses (0.5 ml), and immunosuppressed patients and patients on hemodialysis are given higher doses.

Immunization strategy varies depending on the level of endemicity, with the ideal goal being universal childhood vaccination. Additional strategies include prevention of perinatal HBV infection through routine HBsAg screening of all pregnant women, with immunoprophylaxis given to all babies of HBsAg- positive mothers. As soon as possible after delivery, the infant is given 30 IU of HBIg and an HBV vaccination course is started. Even with prompt administration of HBIg and vaccine, there is still a risk of transmission to the neonate; this risk is increased if the mother is HBeAg-positive and has HBV DNA levels $>10^7$ IU ml^{-1}. In countries with low or intermediate endemicity, a higher proportion of chronic HBV is acquired by older children and young adults, and 'catch-up' vaccination for those who were not vaccinated at birth should be considered, particularly for individuals at increased risk for exposure to HBV, such as healthcare workers and persons engaging in high-risk sexual activity.

HCV

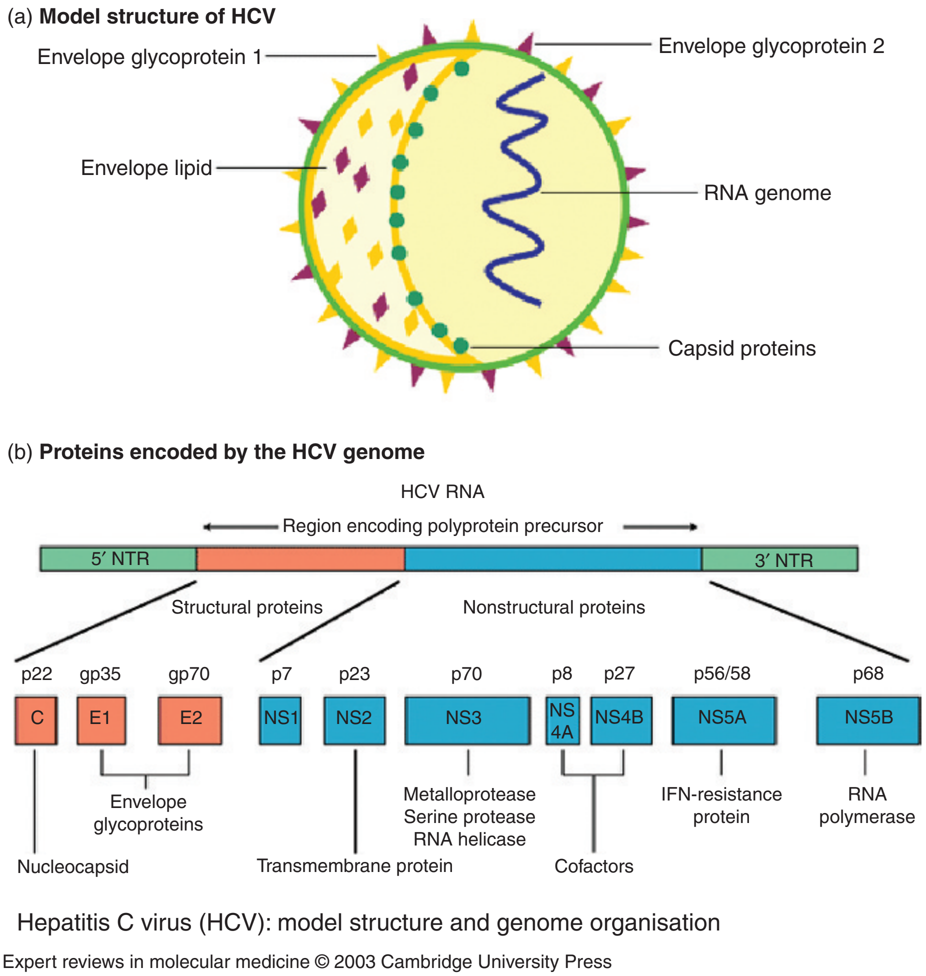
HCV is a parenterally transmitted RNA virus, a characteristic feature of which is a propensity to cause chronic infection, with only 15–20% of acutely infected individuals clearing infection spontaneously. This is explained in part by the extreme genetic diversity of HCV, a feature it shares with other RNA viruses. HCV was first identified in 1989, although clinically its existence had been suspected for many years as the agent responsible for parenterally transmitted 'NANB' hepatitis. HCV is globally distributed, with over 170 million individuals estimated to be infected, and decompensated cirrhosis secondary to chronic HCV is now the leading indication for LT worldwide.

Virology

HCV is a small enveloped positive-sense, single-stranded RNA virus, which is classified as a member of the family Flaviviridae, genus *Hepacivirus*. Within an infected individual, HCV exists as a population of closely related yet distinct 'viral quasispecies,' which may differ with respect to replicative capacity, cell tropism, immunologic escape, and antiviral resistance. HCV has six known genotypes (1 a/b, 2 b, 3a, 4, 5, and 6) and at least 30 subtypes with differing geographical distributions. Genotypes 1, 2, and 3 are distributed worldwide, genotype 4 is found in the Middle East and Africa, genotype 5 in South Africa, and genotype 6 is the predominant genotype in Southeast Asia. Coinfection with more than one genotype is not infrequently seen, and 'superinfection' has been reported.

The genome of HCV contains a single, large ORF flanked by 5′- and 3′-untranslated regions, which codes for a polyprotein of approximately 3000 amino acids, which, when cleaved by viral and host peptidases, produces a number of functional proteins, namely three structural proteins, core, and two envelope glycoproteins (E1 and E2), six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a protein (p7). The viral nucleocapsid comprises multiple copies of the core proteins in complex with genomic RNA (**Figure 6**). Apart from humans, HCV infects only chimpanzees, and characterization of the molecular mechanisms involved in viral replication and immune response to HCV was until recently impeded by the lack of a good *in vitro* model. Alternative tissue culture systems have now been developed, including recombinant HCV envelope glycoproteins, HCV-like particles, HCV retroviral pseudoparticles, and, most recently, cell culture-derived infectious HCV. Although these models have certain limitations, they have greatly advanced the understanding of the early steps of viral infection and host–virus interactions, and have facilitated the development of several new classes of antiviral drugs specific for HCV.

Circulating HCV is physically associated with lipoproteins or antibodies. The major target cell is the hepatocyte, but it is thought that HCV may be able to infect other cells, such as lymphocytes, monocytes, and dendritic cells. Both EI and E2 are essential for entry into the host cell. Within the E2 envelope, glycoprotein sequence hypervariable regions (HVR) have been


(a) **Model structure of HCV**

Envelope glycoprotein 1
Envelope glycoprotein 2
Envelope lipid
RNA genome
Capsid proteins

(b) **Proteins encoded by the HCV genome**

HCV RNA
Region encoding polyprotein precursor
5′ NTR
3′ NTR
Structural proteins
Nonstructural proteins
p22
gp35
gp70
p7
p23
p70
p8
p27
p56/58
p68
C
E1
E2
NS1
NS2
NS3
NS 4A
NS4B
NS5A
NS5B
Envelope glycoproteins
Metalloprotease
Serine protease
RNA helicase
IFN-resistance protein
RNA polymerase
Nucleocapsid
Transmembrane protein
Cofactors

Hepatitis C virus (HCV): model structure and genome organisation

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Figure 6 Structural and genomic organization of the hepatitis C genome. Reproduced from Expert Reviews in Molecular Medicine, 2003, Cambridge University Press.

identified, which differ more than 80% among HCV genotypes, and within subtypes of any one genotype. HVR-1 is a HVR that is important for host cell recognition and attachment. A number of potential entry receptors have been identified, including the tetraspanin CD81, scavenger receptor class B type 1 (SR-B1), heparin sulfate, low-density lipoprotein receptor, and, most recently, claudin-1, a protein involved in the maintenance of cell structures called 'tight junctions' found in several epithelial tissues and most prevalent in the liver. Binding of viral envelope glycoproteins to the hepatocyte cell surface triggers endocytosis of the HCV virion, and the viral nucleocapsid enters the cytosol and is transported to the endosome where uncoating, IRES-mediated translation, and RNA replication occur. Four viral enzymes are needed for RNA replication, including the NS2 autoprotease, the NS3–4 serine protease, the NS3 RNA-stimulated NTPase/helicase, and the NS5B RNA-dependent RNA polymerase. The serine protease and RNA polymerase are currently the focus for the development of antiviral agents; once fully elucidated, entry receptors will also provide an important additional target.

Epidemiology

HCV infection is endemic in most parts of the world, with an estimated overall prevalence of 3%. The virus is predominantly transmitted via the parenteral route, although vertical and sexual transmission is recognized. In about 10% of cases, a source of infection cannot be identified. HCV has be shown to remain viable on surfaces outside the body for up to 16 h, and transmission may occur through sharing of toothbrushes or razors contaminated with infected blood.

Before the introduction of serological tests for HCV, infection with contaminated blood or blood products was a frequent source of infection. Use of contaminated pooled clotting factors led to prevalence rates of over

80% in the hemophiliac population in the West, many of whom were also infected with HIV. Following the introduction of screening of blood donors for infection, the risk of transmitting HCV by blood products is presently at 1/200 000 units distributed. Transmission from nonsterile injecting practices in healthcare settings is no longer a significant risk for acquisition of HCV, although sporadic cases have been reported in association with poor disinfection procedures for invasive medical equipment such as colonoscopes. HCV has been transmitted through tattooing and body piercing in establishments re-using needles between clients, and rarely infection has been reported to have contracted through manicures and pedicures. In developed countries, IVDUs are currently the only group at significant risk for acquiring HCV. Prevalence rates of HCV approach 80% in the IVDU population, with most becoming infected within 1–2 years of embarking upon intravenous drug use. Incidence of new infections can be reduced by harm-minimization strategies such as needle exchange programs. Health professionals are at risk following needlestick exposures from an infected source, as there is no equivalent of HBV Ig, and currently no vaccine is available.

Vertical transmission is uncommon (seen in <5% cases), the risk is proportional to the maternal HCV RNA level, with higher levels carrying greater risk. Higher HCV viral loads are usually seen in individuals coinfected with HIV, and increased rates of vertical transmission are seen in HIV-positive mothers.

The role of sexual transmission is controversial. Most data come from studies with HCV-positive hemophiliacs and their female partners, and indicate extremely low risk. Most authorities do not routinely recommend the use of 'safe sex' practices in HCV sero-discordant heterosexual couples. However, there have been several recent reports from the United States and Northern Europe of outbreaks of acute HCV infection in HIV-positive men who have sex with men. Permucosal transmission of HCV related to high-risk traumatic sexual and drug practices has been implicated as the infection route in these cases.

Although HCV infection is found worldwide, there is marked geographic variance in prevalence and incidence, and also between age groups within individual countries. In countries where IVDU is the major risk factor for HCV acquisition, such as North America, Northern Europe, and Australia, the highest prevalence rates are seen in adults aged 30–49, which would indicate an exposure in early adult life. In countries such as Italy and Japan, the majority of affected individuals are older, reflecting remote inadvertent transmission during medical interventions. In Egypt, prior mass parenteral therapy for schistosomiasis with contaminated needles is responsible for high rates of HCV prevalence in the general population.

Acute Hepatitis C – Clinical Features

Depending on the size of the inoculum, HCV RNA can be detected in serum from 6–7 days to 8 weeks after exposure, with the first biochemical evidence of infection. Approximately 80% of acutely infected individuals fail to clear the virus and develop chronic infection. Clinical factors favoring spontaneous resolution include age <40 years, female gender, and icteric presentation.

Following initial infection, HCV may become established despite the induction of a humoral immune response targeted against various epitopes of the HCV envelope glycoproteins. Strong and persistent proliferative responses of CD4$^+$ T cells and the production of IFN-γ are associated with spontaneous viral clearance, whereas weak or transient responses are associated with progression to chronic infection. The role of CD8$^+$ T cells in controlling acute HCV infection is less clear. It is known that IFN-treated individuals who have cleared HCV have decreased HCV-specific immune responses as compared with those achieving spontaneous clearance. There is some evidence that host genetic factors influence the outcome following HCV infection. Certain HLA class II alleles – HLA-*DRB1***1101-DQB1**0301, *DRB1***1104-DQB1**0301, and *DRB1***0401-DQB1**0301 haplotypes – have been associated with a significantly increased likelihood of viral clearance, predominantly in Caucasian populations.

Chronic Hepatitis C – Clinical Features

Insulin resistance (IR) and the other components of the metabolic syndrome (obesity, hyperglycemia, hypertriglyceridemia, increased blood pressure, and low high-density lipoprotein cholesterol levels) and type II diabetes mellitus are more prevalent in individuals with chronic HCV than in healthy controls or patients with chronic HBV. The natural history of chronic HCV infection is variable, ranging from indolent to rapid progression to cirrhosis. Cohort studies have shown that 20% of individuals will progress to cirrhosis over 20 years.

Increased fibrosis progression is associated with male sex, longer duration of infection, acquisition of infection at age >40 years, excess alcohol consumption, immunosupression, that is, from HIV coinfection or following organ transplantation, and coinfection with HBV, but it does not appear related to HCV genotype. Increased body mass index, visceral adiposity, and coexisting IR and hepatic steatosis may also increase the risk of fibrosis progression, and reduce response to IFN.

Diagnosis

Persistence of HCV RNA in the serum 6 months after initial infection indicates chronic HCV, although there are rare reports of spontaneous clearance up to 2 years after exposure.

Current commercially available tests for the detection of HCV antibody are based on either enzyme immunosorbant assays (EIA), which detect HCV-specific antibodies, or on recombinant immunoblot assays (RIBA). EIA's can diagnose more than 95% of chronic infections but can only detect 50–70% of acute infections. RIBA assays identify antibodies that react with individual HCV antigens and are used as a supplemental test for confirmation of a positive EIA result.

Therapy of Chronic Hepatitis C

Unlike chronic HBV in which there is a 'reservoir' of viral cccDNA in host cells with the potential for viral reactivation, there is no such reservoir in chronic HCV infection, and therefore HCV can potentially be eradicated with therapy. SVR is defined as undetectable serum HCV RNA levels (with sensitive PCR-based assays) 6 months after the completion of therapy. The current 'gold standard' of therapy for chronic HCV is a combination of pegylated IFN-α (Peg-IFN) injected once weekly, together with daily oral weight-based RBV, administered for 24 weeks to most patients with HCV genotype 2 or 3, and for 48 weeks to all other genotypes. Mechanisms of action of IFN-α include the induction of an antiviral state that either protects cells from infection or attenuates the production of viral progeny in already infected cells, and, indirectly, the activation of the host adaptive immune response. Both Peg-IFN and RBV have significant side effects, such as depression, fatigue, and cytopenias, and may be poorly tolerated. Efficacy of treatment is highly dependent on viral kinetics, HCV genotype, and ethnicity, with other factors such as gender, age, degree of liver fibrosis, and presence of HS and IR also playing a role. HCV genotype 1, African-American ethnicity, male gender, age >50 years and, advanced liver fibrosis with or without steatosis on liver biopsy are all negatively associated with treatment response. Although SVR rates with Peg-IFN and RBV are better with genotypes 2 and 3 (75–80%) than with genotype 1 (45–50%), there are now data from multiple clinical trials indicating that rapid viral clearance on therapy (undetectable HCV RNA by 4 weeks) is the best predictor of SVR irrespective of genotype. A prolonged period of aviremia while on therapy is beneficial because it allows a longer time for residual virus-infected cells to be eradicated.

Owing to poor tolerability and low efficacy of current therapy, there is a great interest in developing specific candidate small-molecule inhibitors. Both HCV polymerase and HCV protease inhibitors are in clinical trials.

Vaccines

Currently, no vaccine is available to protect against infection with HCV. Vaccine development has been hampered by the lack of a suitable small animal model with which to assess the immunogenicity and protective efficacy of HCV candidate vaccines. Recent advances in this field involve the use of novel challenge viruses, such as recombinant murine gammaherpes virus 68 (MHV-68), to test the efficacy in mice of candidate human vaccines delivering HCV nonstructural NS3 or core proteins.

In addition, a new approach to inducing strong antiviral immunity by the use of dendritic cells (the most potent antigen-presenting cells) transfected with HCV viral antigens has yielded promising initial results.

HDV

HDV, a defective RNA virus also referred to as Delta agent, infects nearly 20 million people worldwide, with a varying geographical distribution. It is only found in association with HBsAg – either as a coinfection in acute hepatitis B or as a 'super infection' in an individual with chronic HBV infection.

Virology

First identified in 1977, HDV is deemed 'defective' as it is unable to replicate by itself, requiring HBsAg as a protein 'coat' for the HDV genome. HDV virions are 36–43 nm, roughly spherical, enveloped particles with no distinct nucleocapsid structure. The outer envelope contains lipid and all three forms of HBsAg (S, M, and L, but predominantly small). The genome of HDV (cloned and sequenced in 1986) consists of a compact (approximately 1.7 kb in length) circular single-stranded negative RNA molecule, which contains several sense and antisense ORFs, only one of which is functional and conserved. The RNA genome replicates via an RNA intermediate, the antigenome, both of which can function as ribozymes to carry out self-cleavage and self-ligation reactions. A third RNA, complementary to the genome and found in the infected cell, is responsible for synthesis of the delta antigen. To date, there have been three genotypes and two subtypes of HDV have been characterized, but emerging data suggest that the genetic variability of the HDV genome is more complex than previously thought. Genotype 1 is mainly found in North America, Asia, Middle East, and Europe; genotype 2 in East Asia; and genotype 3 in South America. Genotype 2 is thought to cause less severe disease than the other two genotypes.

HDV genomic replication is not acutely cytopathic, and does not occur in cells other than hepatocytes; hence, the liver is the only organ affected. HBV is essential for the evolution of the hepatocellular necrosis and inflammation seen with HDV infection. Both humoral and cellular immune mechanisms are thought to be involved.

Epidemiology

Like HBV, HDV is transmitted by blood-borne and sexual routes, but in contrast perinatal transmission is rare. Infection with HDV has a global distribution, with two distinct epidemiologic patterns. First, high prevalence areas for HDV coincide with certain areas of high prevalence for chronic HBV infection, including the Mediterranean, Middle East, Central Africa, and the Amazon Basin in South America (**Figure** 7). In these areas, viral transmission takes place predominantly by non-percutaneous means, especially close personal contact. It is not known why HDV coinfection is not seen in other countries with a high prevalence of HBV, such as Southeast Asia and China. The second pattern is of parenteral transmission in subjects with multiple parenteral exposures, primarily injection drug users and hemophiliacs, as is seen in nonendemic areas such as Northern Europe and the United States. Worldwide, HDV infection is declining. Even in Italy, an HDV-endemic area, public health measures introduced to control HBV infection there resulted in a significant reduction in the prevalence of HDV infection.

Clinical Features

The outcome of infection with HDV depends on whether HDV and HBV infect simultaneously (coinfection) or if HDV is acquired by an individual with chronic HBV (superinfection). Mortality from HDV infection is 10 times higher than for HBV alone.

In coinfection, both acute HBV and HDV hepatitis occur. Whether one or two bouts of clinical hepatitis are seen depends on the relative titers of HBV and HDV. The incubation period of acute HDV is between 3 and 7 weeks, with a pre-icteric phase lasting 3–7 days, during which time the classic symptoms of acute hepatitis are manifested (fatigue, lethargy, anorexia, and nausea), and ALT and AST become elevated. This phase is followed by jaundice, which can last for several weeks. The severity of the resulting illness is typically greater than with HBV monoinfection, with a higher incidence of fulminant hepatitis, and low risk of chronic infection (<5%). During the acute phase of HDV infection, synthesis of both HBsAg and HBV DNA is suppressed until HDV is cleared.

Hepatitis D superinfection is frequently associated with severe acute hepatitis with progression to chronic HDV in up to 80% of cases. Fulminant hepatitis also occurs. Superinfection can transform inactive or mild chronic hepatitis B into severe, progressive chronic hepatitis, and result in faster progression to cirrhosis than seen with chronic HBV alone. Up to 70% of patients with chronic HDV will develop cirrhosis.

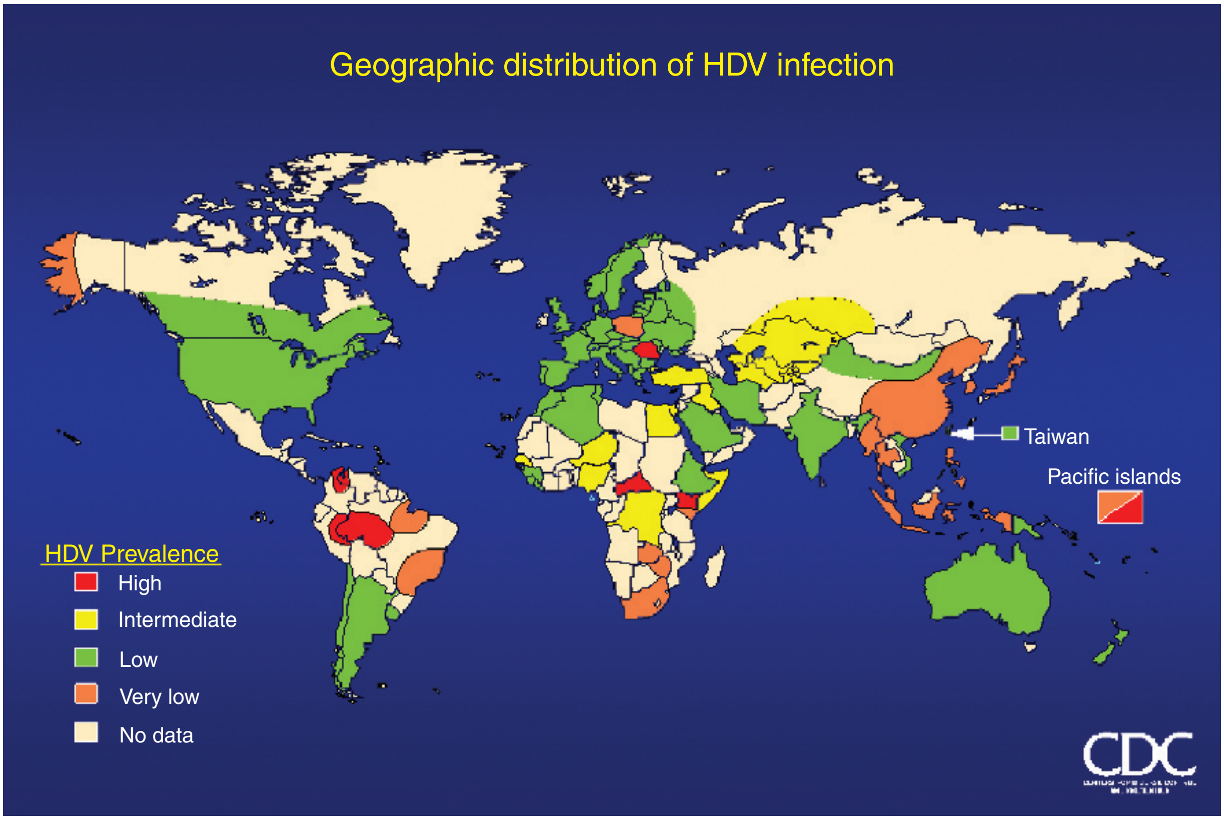

Figure 7 Geographic distribution of hepatitis D. Reproduced from Center for Disease Control and Prevention, USA.

Diagnosis

Anti-HDIgM, HDV RNA, and HDAg are the most sensitive markers for the diagnosis of acute HDV, although anti-HDIgG is the only test commercially available. In acute HBV–HDV coinfection, HBsAg precedes the appearance of HD Ag and HDV RNA. Anti-HDIgM develops late in the acute phase, and is accompanied by the disappearance of HDAg. In about 15% of patients the only evidence of HDV infection may be the detection of either IgM anti-HDV alone during the early acute period of illness or IgG anti-HDV alone during convalescence. Both IgM and IgG anti-HD antibodies decline after recovery to subdetectable levels within months to years, and it can sometimes be difficult to demonstrate past HDV infection as there is no serologic marker that persists to indicate that the patient was ever infected with HDV. These immune responses are thought to be protective, as second cases of HDV have not been reported.

With HBV–HDV superinfection, HBsAg titers decline with the appearance of HD-Ag in the serum. HDV RNA levels reach a peak between 2 and 5 weeks after exposure, declining over a period of 1–2 weeks. High titers of IgM and IgG anti-HD are detectable in the acute phase, and can persist for months or even indefinitely. Most superinfections result in chronic HDV, demonstrated by the persistence of HD-Ag, HDV RNA, and high levels of IgM and IgG anti-HD. HDV viremia is associated with active liver disease.

Therapy

High-dose IFN-α (up to 9 million units three times weekly for 48 weeks) has been evaluated in individuals with chronic HDV, but does not achieve viral eradication in the majority of those treated, although improvement in biochemical and histological parameters has been observed. Treatment of the associated HBV infection with oral antiviral drugs does not alter the course of HDV infection.

Vaccines

No vaccine is available specifically for Delta virus; however, as this virus is only acquired in association with HBV, vaccination against HBV will also prevent against the transmission of HDV. HBIg will not protect individuals with chronic HBV from infection with HDV.

HEV

HEV is an enterically transmitted RNA virus responsible for outbreaks of hepatitis in developing countries and sporadic cases of acute hepatitis in endemic areas such as Southeast and Central Asia (**Figure 8**).

Virology

Although recognized as a clinical entity in the 1980s, the virus responsible for acute enterically transmitted NANB hepatitis was not identified until 1983. HEV has recently been classified in the genus *Hepevirus* of the family Hepeviridae.

Cloned in 1990, the HEV genome is a nonenveloped, single-stranded, positive-sense RNA molecule 7.2 kb in length, with three ORFs. ORF 1 codes for viral enzymes methyltransferase, proteases, helicase, and replicase; ORF 2 codes for the major HEV capsid protein, the function of the protein coded for by ORF 3 has not yet been well characterized, although it appears to be involved in virus–host interactions. Four major genotypes have been identified, geographically distinct, but all share at least one major serologically cross-reactive epitope despite substantial genomic variability, which is an important observation that may facilitate the development of a protective vaccine.

Viral replication, transcription, virus–host interactions, and pathogenesis of HEV are poorly understood due to the lack of a cell culture system and a practical animal model for HEV. Subgenomic expression strategies

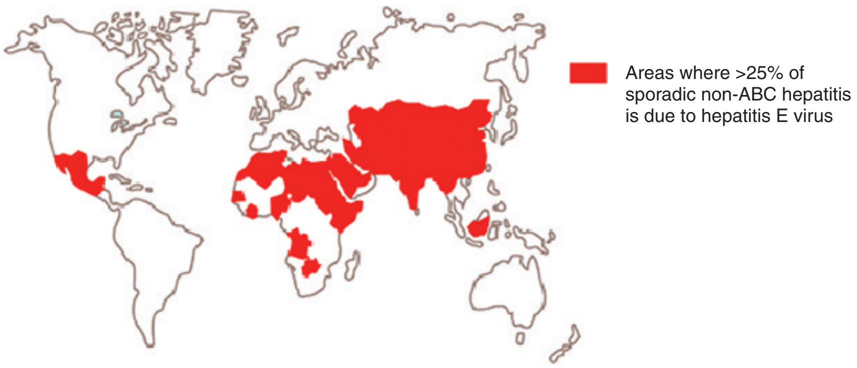
🟥 Areas where >25% of sporadic non-ABC hepatitis is due to hepatitis E virus

Figure 8 Geographic distribution of hepatitis E. Reproduced from Expert Reviews in Molecular Medicine, 2003, Cambridge University Press.

have been used to fractionally characterize HEV-encoded proteins. However, in common with other hepatitis viruses, the mechanism of liver injury is probably immunological, as the onset of ALT elevation and histopathological findings of acute hepatitis correspond with the appearance of antibodies to HEV and decreasing levels of HEV antigen in hepatocytes.

Epidemiology

Outbreaks of acute HEV infection affecting several thousand people have been reported from Asia, the Middle East, Northern and Western parts of Africa, and Central America. HEV is transmitted through the fecal–oral route and most epidemics have been associated with contaminated drinking water. Although like HAV, HEV is detectable in the stools of infected individuals, person-to-person transmission is less commonly observed. Materno-fetal transmission is thought possible, and there have been isolated case reports of parenteral transmission. Sexual transmission is debatable.

HEV is a common cause of sporadic hepatitis in endemic areas, accounting for almost 50–70% of acute hepatitis cases in India. In industrialized nations, acute HEV infections are infrequent, accounting for <1% of cases of acute viral hepatitis, and are usually associated with a history of travel to an endemic area. However, a number of reports of nontravel-associated HEV have been recently reported from Europe, United States, and Japan, and in these cases it has been postulated that HEV may have been acquired from a viral reservoir in swine or possibly rodents. In support of this theory, high seroprevalence of anti-HEV has been reported in pig veterinarians, and farm workers in contact with pigs. In Japan, cases of acute infection associated with the consumption of inadequately cooked pig liver have been reported. HEV may therefore be viewed as a new emerging pathogen with zoonotic potential.

There is evidence that subclinical infection may be common in endemic areas; one study reports HEV seroprevalence rates of 30–50% in healthy subjects. Another study from Southeast Asia followed up a cohort of healthy individuals over an 18-month period and found that 66% of those who seroconverted to anti-HEV had no history of jaundice, suggesting that such ‘subclinical’ cases may act as reservoirs for infection in endemic populations and also as a source for cases of sporadic acute HEV.

Clinical Features

As for hepatitis A, the attack rate is highest for young adults aged between 15 and 40. Work with susceptible primate models and infected individuals reveals that the incubation period is approximately 21 days, with the expression of HEV antigen in the serum and stool occurring 7 days before the onset of symptoms, and persisting for approximately 2 weeks. ALT and AST begin to rise a few days before the onset of symptoms, but generally return to normal within 1–2 months after the peak severity of the disease has passed. Anti-HEV (IgM) appears shortly after the onset of clinical illness and persists only for a few months. IgG anti-HEV appears a few days after IgM and persists for up to 3 years, and may afford protection against reinfection.

Clinical features are similar to other acute viral hepatitides. Acute HEV infection is self-limiting, and does not become chronic. However, HEV can cause fulminant liver disease in individuals with underlying cirrhosis and pregnant women, particularly during the third trimester of pregnancy when mortality rates of up to 25% have been reported. Disease severity may correlate with HEV genotype. In Japan where 3 and 4 are the most prevalent HEV genotypes, two studies found that patients with genotype 4 experienced higher peak ALT levels and longer hospital stays as compared with patients with genotype 3.

Diagnosis

Infection with HEV can be confirmed by the detection of anti-HEV antibodies or the presence of HEV RNA in the serum detected by RT-PCR. Simultaneous detection of IgA and IgM antibodies against HEV is highly specific for the diagnosis of acute infection.

Vaccines

There are no commercially available vaccines against HEV, although encouraging results have been reported from recent clinical trials. In 2007, Shrestha and colleagues evaluated recombinant HEV vaccine in (mostly male) volunteers in the Nepalese Army; 896 subjects in the vaccine group and 898 in the placebo group received three doses (at 0, 1, and 6 months) of recombinant HEV capsid protein or saline, respectively. The protective efficacy against clinically overt HEV infection was 95.5% in subjects receiving all three vaccine doses and 85.7% after two doses. The vaccine was well tolerated. This study clearly showed that a recombinant HEV vaccine effectively prevents clinical HEV hepatitis.

‘New’ Hepatitis Viruses

Several recently discovered viruses, namely Torque teno virus (TTV), SEN-V, and hepatitis G, although potentially hepatotropic, have yet to be conclusively shown to be pathogenic to the liver.

In 1994, an agent (initially named hepatitis F virus) was isolated from a stool sample and found to be transmissible in primates. Subsequent researchers have not been able to confirm the existence of this agent, and there is currently no confirmed HFV.

TTV

TTV, family Circoviridae, genus *Anellovirus*, has a single stranded circular DNA genome. Related viruses are also found in primates, chickens, pigs, cows, sheep, and dogs. This virus was first identified in 2001 in a patient presenting with non-A–E acute recurrent hepatitis with no history of drug or alcohol abuse and negative markers of viral or autoimmune disease. Transmitted parenterally and probably also enterically, the TTV family of viruses is present in over 90% of adults worldwide. Human pathogenicity, particularly regarding the liver, has yet to be clearly established.

SEN Virus

Named after the initials of the patient with HIV from which it was first isolated, SEN virus (SENV) is a novel circular single-stranded DNA virus distantly related to TTV and also classified within the family Circoviridae, which has been associated in some studies with post-transfusion hepatitis. Variants designated A–H have been identified, which have differing geographical prevalence, for example, SENV-D is highly prevalent in Japan.

HGV

In 1995 and 1996 several new flaviviruses, GB-A, GB-B, and GB-C, related to but distinct from HCV, were identified. GB-A and GB-B infect tamarin monkeys but GB-C can infect humans and has been implicated in some cases of acute and chronic hepatitis. Another group identified a virus which they termed HGV; based on genomic sequence comparisons, HGV and GB-C can be considered the same virus.

HGV is a positive-stranded RNA virus, classified in the family Flaviviridae, which has at least four major genotypes. It is thought to be lymphotropic, replicating predominantly in the spleen and bone marrow. Molecular epidemiological studies have demonstrated that about 1–1.4% of the healthy population in developed countries are chronically infected with HGV. Parenteral is the most frequent mode of infection, and HGV has been implicated in a small number of cases of post-transfusion hepatitis. There is also a higher prevalence of HGV in drug users and patients on hemodialysis. Whether HGV is a significant cause of human hepatitis is still unconfirmed. Although relatively common, infection with HGV may cause a mild acute hepatitis but does not seem to cause clinically significant chronic liver disease. In one US study evaluating causes of acute viral hepatitis, HGV RNA was detected in 9% of patients with acute non-A–E hepatitis, 20% of those with HCV, 25% of those with HAV, and 32% of those with HBV. Another study examined serum samples from blood donors, transfusion recipients and controls. Prevalence of HGV in the nontransfused individuals ($n = 657$) was 1.4%. There were 35 HGV infections among the 357 transfusion recipients, with only three having HGV as the only agent detected. All these subjects had a mild hepatitis and did not develop clinically apparent chronic hepatitis (although liver biopsy was not performed).

A French study retrospectively examined 228 subjects with chronic HCV for HGV coinfection; 21% of the cohort and 32% of the subset who were IVDUs were coinfected. Another French study found that 57.5% of a cohort of 61 hemodialysis patients tested positive for HGV RNA; however, only four of these subjects had viremia associated with elevated serum ALT.

Several studies in HCV coinfected individuals, although with small numbers of subjects, have established that severity of liver disease, serum HCV RNA levels, and response to IFN therapy are not influenced by HGV coinfection. HGV RNA levels were observed to decrease with IFN therapy, but most subjects became detectable again once IFN was discontinued.

Future Prospects

Prospects for lessening both mortality and the socioeconomic burden resulting from viral hepatitis are optimistic, because safe and effective vaccines are currently commercially available for HAV and HBV, and a vaccine for HEV appears imminent. Improvements in sanitation and dissemination of simple public health information will lessen the transmission of HAV and HEV in endemic areas. Pretransfusion screening of blood and blood products for infectious agents is now routine in the majority of developed countries, thus lessening the iatrogenic transmission of HBV, HDV, and HCV. In Asia, where vertical transmission of HBV constitutes the major route of infection, increasing maternal HBV screening, and use of both active (administration of HBV vaccine) and passive (administration of hyperimmune globulin) immunoprophylaxis for infants at high risk will prevent most neonatal infections, leading to a decreased incidence of chronic HBV-related complications (such as HCC) in these populations over time. Owing to particular viral characteristics, development of a vaccine for HCV remains difficult with no current indication of when such a vaccine might be available. Fortunately, the incidence of HCV, at least in the Western world, is declining because of routine screening of blood and blood products before transfusion, and the implementation of risk minimization strategies, for example, needle exchange programs, in populations such as IVDUs.

In both chronic HBV and HCV, achieving viral eradication with currently available therapies is problematic.

Nevertheless, new classes of specific antiviral drugs are moving quickly from 'bench to bedside' and will likely be used in novel treatment strategies, such as combinations of two or more drugs with or without immunomodulatory therapy such as IFN, with potential improvement in response rates.

Many cases of acute hepatitis occur without any identification of a causative agent, viral or otherwise, which would imply that there are additional pathogenic hepatotropic agents. The search for these agents will provide a continuing diagnostic and therapeutic challenge.

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Herpesviruses

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Defining Statement
Family *Herpesviridae*
The *Alphaherpesvirinae* Subfamily
The *Gammaherpesvirinae* Subfamily
The *Betaherpesvirinae* Subfamily
Further Reading

Glossary

antiviral drugs A class of medication used specifically for treating viral infections. As with antibiotics for bacterial infections, specific antivirals are used for specific viruses.

immunocompetent An individual with a normal immune system; the individual is capable of developing an immune response to an infection.

immunocompromised An individual whose immune system is compromised in some way, the individual lacks the ability to mount a normal immune response to an infection and is often unable to resist or fight off infection.

latency A quiescent period of infection when most or all of the viral lytic genes are silenced, and the virus is not making progeny virus.

lytic A type of infection in which viral lytic genes are switched on and the virus is actively undergoing replication and making progeny virions. This is also referred to as a productive infection.

seropositive A situation where an individual has antibodies against a certain pathogen in their blood, being seropositive indicates that the individual was previously exposed to that pathogen.

vaccine A preparation that contains an antigen, consisting of an organism or a part of an organism, that is used to confer immunity against the disease that the organism causes. Vaccines can be natural, synthetic or derived from recombinant DNA technology.

Abbreviations

ACV	acyclovir
BL	Burkitt's lymphoma
CD8$^+$	cluster of differentiation 8
CD21	complement receptor 2
E genes	Early genes
EBNA	EBV-coded nuclear antigens
EBV	Epstein-Barr virus
CAEBV	chronic active EMV
ES	exanthem subitum
NHANES	National Health and Nutrition Examination Survey
HPC	hematopoietic progenitor cells
HSE	herpes simplex encephalitis
HSV	herpes simplex virus
HSK	herpetic stromal keratitis
HCF	host cell factor
HCMV	human cytomegalovirus
HHV4	human herpesvirus 4
HHV-6	Human herpesvirus 6
HHV8	human herpesvirus 8
HHV	Human herpesvirus
IE genes	immediate early genes
IM	infectious mononucleosis
IFN-γ	interferon-γ
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L genes	Late genes
LATs	latency-associated transcripts
LMPs	latent membrane proteins
LCV	*Lymphocryptovirus*
MHC	major histocompatibility complex
MCMV	models with murine CMV
NK cells	Natural killer cells
NPC	nuclear pore complexes
Oct-1	octamer binding protein 1
PHN	post-herpetic neuralgia
PTLD	posttransplant lymphoproliferative disease
PEL	primary effusion lymphoma
RDV	*Rhadinovirus*

TK	thymidine kinase	**VAHS**	Virus-associated hemophagocytic syndrome
VZV	varicella-zoster virus	**XLP**	X-linked lymphoproliferative
VP16	viral protein number 16		
VHS	virion host shutoff		

Defining Statement

The family *Herpesviridae* includes over 100 different species of DNA viruses, eight of which are currently known to infect humans. These viruses are discussed with relation to the diseases they cause, their ability to establish latent infections, their biology, replication and pathogenesis, and the treatment options available for each.

Family *Herpesviridae*

The family *Herpesviridae* is a family of large DNA viruses containing over 100 different virus species that infect hosts ranging from humans to birds to reptiles. Classification of a virus as a member of the family *Herpesviridae* is based on a shared virion structure: a linear, double-stranded DNA genome is contained within a central core, surrounded by an icosahedral capsid. This capsid is in turn surrounded first by an amorphous protein layer, known as the tegument, and then by an envelope containing viral glycoprotein spikes (**Figure 1**). Herpesviruses also share four significant biological properties:

1. They encode a large number of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins.
2. The synthesis of viral DNAs and caspid assembly occurs in the nucleus of the infected cell. During infection, virus-specific compartments are assembled within the nucleus of the infected cell, commonly referred to as replication compartments (**Figure 2**). It is within these compartments that viral DNA replication, late viral gene expression, and encapsidation of progeny viral genomes occur. These compartments lead to the formation of basophilic nuclear inclusion bodies, which are diagnostic of herpes virus infection.
3. Production of infectious progeny virus is generally accompanied by the destruction of the infected cell.
4. The viruses are able to establish a latent infection in their natural hosts.

There are currently eight herpesviruses that are known to infect humans: herpes simplex virus (HSV)-1 and HSV-2, human cytomegalovirus (HCMV), varicella zoster virus (VZV), Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) and human herpesvirus (HHV)-6 and HHV-7. These viruses, along with the majority of herpesviruses that infect other mammals and birds, have been divided into three subfamilies, alpha, beta, and gamma, based on the biological properties of the viruses. HSV-1, HSV-2, and VZV are members of the *Alphaherpesvirinae* subfamily, EBV and KSHV are both members of the *Gammaherpesvirinae* subfamily, while the remaining viruses, HCMV and HHV-6A, HHV-6B, and HHV-7 are all members of

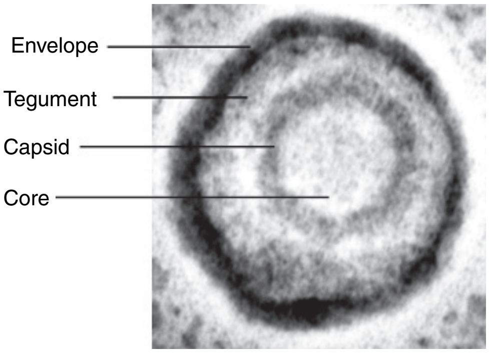

Figure 1 Structure of the herpes simplex virus (HSV) virion. An electron micrograph of a negative-stained HSV-1 virion. The envelope, tegument, capsid, and core are indicated. Micrograph provided by Dr. Travis Taylor.

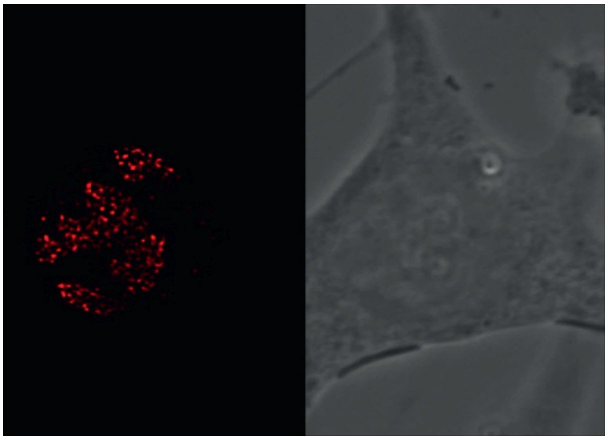

Figure 2 Replication compartments in the nucleus of a herpes simplex virus (HSV)-infected cell. Shown is an immunofluorescence image illustrating the localization of ICP8 to replication compartments within the nucleus of an infected cell. ICP8 staining is shown on the left, the location of the nucleus within the cell can be seen on the right. Figure provided by Lindsey Silva.

the *Betaherpesvirinae* subfamily. Despite the many similarities in structure and biological properties shared by herpesviruses, it is not surprising that in a group of this size there are also many differences. Host range, length of replicative cycle, cell type in which latency is established, and clinical manifestations of disease all vary among the different members of the family.

The *Alphaherpesvirinae* Subfamily

Members of the *Alphaherpesvirinae* subfamily are characterized by a variable host range, short reproductive cycles, the ability to spread rapidly in culture and efficiently destroy infected cells, and the capacity to establish latent infections primarily, although not solely, in sensory ganglia. The members of the *Alphaherpesvirinae* subfamily that infect humans are HSV-1 and HSV-2, and VZV.

HSV

Disease

The two HSV species, HSV-1 and HSV-2, are capable of causing a variety of diseases within an infected host. The most common of which are orolabial lesions, commonly referred to as coldsores or fever blisters and most often caused by HSV-1, and genital herpes which is most often caused by HSV-2. The viruses are extremely widespread throughout the world’s population. The US National Health and Nutrition Examination Survey (NHANES) conducted between 1999 and 2004 has put seroprevalence rates in the United States at 58% for HSV-1 and 17% for HSV-2. Seroprevalence rates in Europe tend to be slightly higher for HSV-1 and slightly lower for HSV-2 when compared to the United States, although there are large intercountry differences. In developing Asian countries, the seroprevalence of HSV-1 and HSV-2 appear to match those seen in Europe and the United States. However, in Africa and Central and Southern America, the picture is quite different. In these regions, HSV-1 is becoming an almost ubiquitous pathogen with greater than 90% of the population seropositive by their fourth decade of life. HSV-2 seroprevalence rates range from 30 to 80% in women and 10 to 50% in men in sub-Saharan Africa, and between 20 and 40% in women in Central and South America. Universally, HSV-2 seropositivity is higher in women than in men. Changes in sexual practices also mean that HSV-1 is becoming a more common cause of genital infection than it once was.

In an immunocompetent host, primary infections with HSV can be asymptomatic, with an individual only realizing that they have been infected when a recurrent infection occurs at a later point. However in some cases, primary infections can be symptomatic, and in these instances disease is usually more severe than that seen with recurrent infection. During symptomatic primary HSV infection, individuals can present with fever, malaise, and large quantities of painful vesicular lesions at and around the site of infection, lasting for a period of up to 3 weeks. Recurrent infections, at either the orofacial or genital site, generally involve a much smaller number of vesicular lesions that persist for 7–10 days.

Along with the common mucosal herpetic lesions associated with orofacial and genital infections, HSV is also associated with a number of more severe complications. In immunocompetent individuals, the most serious complications are herpetic stromal keratitis (HSK) and herpes simplex encephalitis (HSE). Ocular infection with HSV can lead to HSK, the leading cause of infectious corneal blindness. Initially, recurrent infections within the cornea can produce ulcers that result in pain, light sensitivity, and blurred vision. Repeated episodes of recurrent disease can lead to involvement of the underlying stroma, resulting in HSK, which can eventually lead to blindness due to corneal scarring and vascularization. HSE, while extremely rare, has a high risk of mortality if left untreated (>70%). It is usually caused by HSV-1 and results in inflammation and swelling of the brain tissue, with patients presenting with weakness, visual disturbances, and seizures. Although antiviral drugs can be used to decrease mortality, almost 50% of patients fail to regain complete normal function.

As is common with herpes viruses, individuals with compromised or absent immune responses are at high risk of HSV complications. Patients with atopic dermatitis, where the immune response in the skin is skewed toward a Th2 response, can develop a disseminated HSV infection throughout the skin known as eczema herpeticum. Evidence from HIV-positive patients and people undergoing immunosuppressive therapy has demonstrated the increased severity of HSV infection in such populations, with these patients also more prone to chronic or atypical infections. Finally, HSV infection in neonates is associated with increased mortality and morbidity. Infection in this setting usually results from transmission from mother to child during delivery and is estimated to occur at a rate of one in every 3–5000 deliveries. Localized infections of the skin, eyes, and mouth are rarely fatal. However, children with disseminated infections or those involving the central nervous system are at high risk of mortality or ongoing neurological impairment. Prompt antiviral treatment has been able to reduce mortality rates; however, neurological sequelae remain high in children who recover from either disseminated HSV or HSV encephalitis.

Virus and biology

The genome of HSV-1 is 152 kbp in length, while that of HSV-2 is 154 kbp. The two viruses have approximately 50% nucleotide sequence identity and encode protein products with high levels of amino acid sequence identity. Both viruses have the same genome structure of a unique long and unique short region flanked by inverted repeats and share the common herpesvirus virion structure described previously. The high level of shared protein sequence results in antigenic cross-reactivity between the two viruses but despite this they have different neutralization patterns and tend to produce different clinical symptoms.

One biological property, common to both HSV-1 and HSV-2, that influences the ability of these viruses to cause disease in humans is neurovirulence. The ability of the virus to invade and replicate within the host nervous system primarily enables the virus to establish a latent reservoir of virus within this site, from which reactivation and subsequent transmission can occur. However, it also provides a situation whereby the virus can produce severe disease within the host, such as is seen in cases of HSV encephalitis.

Replication

In permissive cells, the process of HSV viral replication takes 18–20 h (**Figure 3**). The initial step in this process is the attachment to and entry of the virus into the target cell, a process that involves five viral glycoproteins – gB, gC, gD, gH, and gL. The initial attachment is mediated by contact between glycoprotein C (gC) and/or gB with heparan sulfate on the surface of the cell. Viral attachment is closely followed by interaction between gD and one of the several cell surface receptors that facilitate entry into the cell; three different classes of receptors have been identified for gD. Viral entry can occur via two pathways, the well-characterized method of direct penetration of the cellular membrane via fusion with the viral envelope and the less well-characterized method of endocytosis. This second method of entry has to date only been demonstrated using *in vitro* systems with several different cell lines. Hence, the importance of endocytosis in a natural infection remains to be determined. The kinetics of both modes of viral entry appears to be similar, with the transition from attachment to penetration occurring within minutes.

Following entry into the cell, the de-enveloped viral capsid is transported through the cytosol to the nuclear pore complexes (NPC) where the viral DNA is released into the nucleus and takes on a circularized form. Movement of the capsid through the cytosol is rapid, reaching the nucleus within approximately 1 h. This transport is most likely mediated by microtubules. Once the viral DNA has entered the nucleus, the host RNA polymerase II is then used to transcribe the viral genome in a sequential fashion, resulting in the expression of over 80 viral proteins. The viral proteins are preferentially translated within the infected cell, in part due to the action of the viral protein VHS (virion host shutoff).

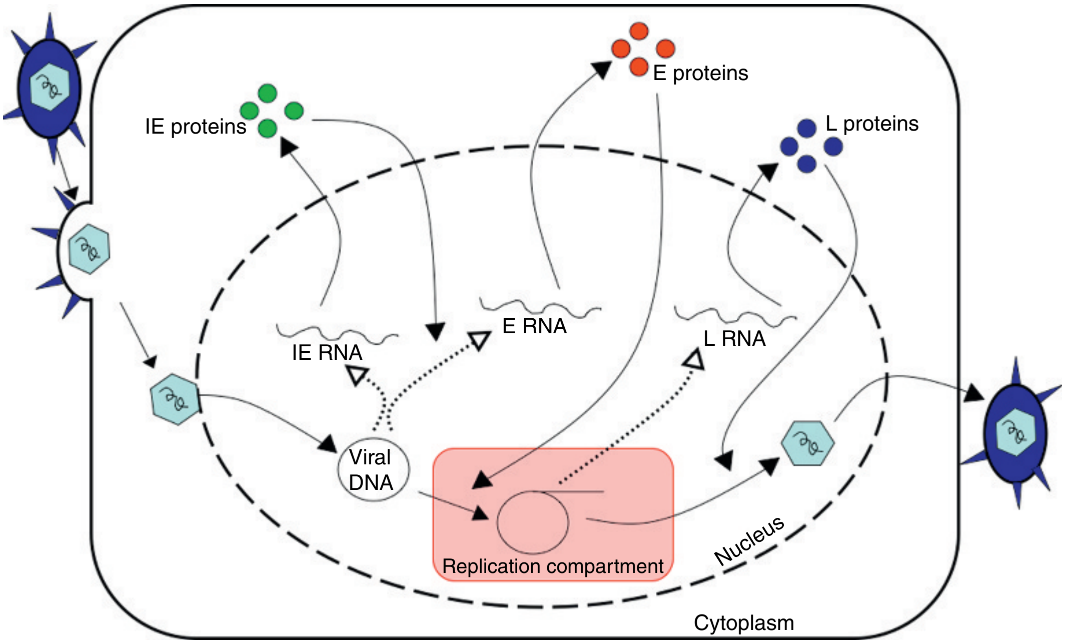

Figure 3 Replication cycle of herpes simplex virus (HSV). The virus binds to the cell surface and the capsid and tegument are released into the cytoplasm following fusion of the viral envelope with the plasma membrane. The capsid is transported to the nuclear pore; the viral DNA is released into the nucleus and becomes circularized. The host RNA polymerase II transcribes the immediate early (IE) genes; the mRNAs are transported into the cytoplasm where translation occurs. The IE proteins activate transcription of the early (E) genes that are involved in viral DNA replication. Viral DNA replication occurs within replication compartments and stimulates transcription of the late (L) genes. The L proteins are involved in assembling the capsids within the nucleus. Progeny viruses exit from the cell by one of three potential mechanisms described below.

This tegument protein remains in the cytoplasm as the viral capsid is transported to the nucleus. It induces destabilization of host mRNA and causes a rapid cessation of host protein synthesis, resulting in the loss of the cellular mRNA pool and thus, the preferential translation of viral proteins. The sequential pattern of viral gene expression results in the HSV genes being divided into three categories, loosely based on the timing of their expression in infected cells. The categories are the immediate early (IE) or α genes, the early or β genes, and the late (L) or γ genes.

IE genes

The IE genes are expressed within 2–4 h of infection and include six different viral proteins. These genes can be expressed in the absence of *de novo* viral protein synthesis, using the viral tegument protein VP16 (viral protein number 16) as a transactivator. VP16 is transported to the nucleus with the cellular host cell factor (HCF) protein. Once within the nucleus, the VP16–HCF complex binds to a second cellular factor, Oct-1 (octamer binding protein 1) and forms a stable transcription regulatory complex called the VP16-induced complex. This complex is able to stimulate expression of the IE genes.

The IE genes ensure the orderly expression of subsequent viral genes and the evasion of the cellular responses to the infection. To do this, many of the IE genes perform multiple functions, and a variety of posttranslational processing is employed to enable these proteins to fulfil the roles required. In brief, ICP4 is required for the expression of all early (E) and L genes; ICP0 acts as a nonspecific or ‘promiscuous’ transactivator, capable of stimulating the transcription of α, β and, γ genes; ICP27 is required for the transcription of the L viral genes and some E genes; ICP47 is involved in immune evasion; and ICP22 and $U_S1.5$ are thought to be involved in promoting the expression of some L genes.

E genes

The E genes are maximally expressed between approximately 5–7 h after infection. The E proteins include those that make up the viral DNA replication machinery. Thus, the expression of the E genes signals the initiation of viral DNA replication. However, the expression of certain E genes is also involved in downregulating IE gene expression, in particular, ICP8 has been shown to downregulate expression of the IE gene ICP4.

L genes

The L genes are the final group of viral genes to be expressed within the infected cell. Some L genes, such as gB and gD, are actually expressed early in the infected cell, and their expression simply increases with the onset of viral DNA replication. Alternatively, other L genes, including gC and U_S11, are only expressed following viral DNA replication. Many of the L genes encode viral structural proteins, and their expression enables the production of progeny virion particles.

Viral egress from infected cells

Following the expression of the L genes, viral capsids are assembled within the nucleus. These capsids, predominantly made up of four viral proteins, are then filled with viral DNA, a process that utilizes viral proteins. It is generally accepted that the nucleocapsids then bud through the inner nuclear membrane and upon doing so acquire an envelope. However the subsequent events that lead to the egress of the newly formed virus particle from the infected cell are not yet fully understood. Three competing theories exist, each with varying amounts of supporting evidence. The first theory argues that the enveloped nucleocapsid buds through the inner nuclear membrane and is transported to the surface by vesicular movement through the Golgi apparatus; thus, in this model, the tegument would be acquired in the nucleus. The second theory argues that the enveloped virus fuses with the outer nuclear membrane, leaving the de-enveloped nucleocapsid to bud into the Golgi apparatus, regaining an envelope, and then to travel to the surface via vesicular movement. In this model, the tegument could be acquired in the nucleus or the cytoplasm, with work supporting this model demonstrating that the majority of virions gain their tegument and envelope in the cytoplasm. The third model involves capsids exiting the nucleus via nuclear pores and then budding through Golgi membranes.

Release of virus from an infected cell results in the shedding of newly formed virions, enabling the virus to spread to susceptible individuals. However, within the body, the virus can also spread directly from cell to cell. This process involves the viral glycoproteins gE and gI, which form a heterodimer, and is facilitated by cell contact. In general, cells infected with replicating HSV do not survive the infection, due to the cytopathic effects of viral infection.

Latent infection

HSV persists for the life of the host by establishing a latent infection in sensory neurons. During a primary infection, virus enters the sensory nerve endings in the epithelium and travels to the neuronal cell body. Animal models have suggested that within the neuronal cell body viral replication can occur initially. However, within several days, no replicating virus can be detected. Concurrent with the short-lived lytic infection within the ganglia, the virus also establishes latency and, following clearance of the replicating virus, latent virus persists. Evidence from animal models and human studies has indicated that the latent HSV genome most likely exists as an extrachromosomal circular episome, with human studies suggesting a latent viral burden of between 1 and 10 viral copies per neuron, remembering that not all neurons within a ganglion will harbor latent virus. Viral replication within the nerves or even at the primary site of infection is not required for the establishment of latency. However, lack of viral replication appears to reduce the quantity of latent virus within a latently infected ganglion through a reduction in the number of latently infected cells.

The traditional view of latency is that lytic viral gene expression is shut down and only the latency-associated transcripts (LATs) are produced. The primary LAT is an 8.5 kb transcript that is cleaved to produce the 2.0 kb and 1.5 kb major LATs, referred to as such based on their abundance. LATs may play a role in silencing lytic genes, preventing cell death, or exerting other effects during latent infection.

As mentioned previously, latent virus can reactivate periodically. It is thought that only a small percentage of latently infected neurons will reactivate at any given time. Following reactivation within the ganglion, viral components such as the nucleocapsid and the glycoproteins travel down the neuronal axon individually via anterograde axonal flow and are assembled into virus particles prior to virus emergence into the periphery. Within the periphery, reactivated virus can result in either asymptomatic shedding or a clinical recurrent lesion. Although the mechanisms are not fully understood, a number of stimuli are known to induce reactivation including nerve damage, stress, ultraviolet (UV) light, menstruation, and hormonal imbalances. The fate of virus-infected neurons continues to be contentious, with some believing that neurons can survive a lytic HSV-1 infection and others arguing that they cannot.

Pathogenesis

Transmission of HSV requires close, personal contact between the susceptible individual and an individual secreting the virus, enabling the virus to come into contact with either mucosal surfaces or abraded skin. As previously mentioned, HSV-1 is generally the cause of orofacial infections while HSV-2 is usually transmitted via genital contact, and thus causes genital infections, although it should be noted that both viruses are capable of infecting either sites. At each site, the virus replicates in the epithelium and infects the innervating sensory nerve endings. Virus travels along the neuronal axon to the innervating sensory ganglion, the trigeminal ganglion in orofacial infections, and the sacral ganglia in genital infections. Within the sensory ganglion, HSV establishes a lifelong latent infection in which viral gene expression is silent except for transcription of the LAT.

A major factor in the pathogenesis of HSV is the ability of the virus to reactivate from latency. Although we are yet to fully understand the mechanisms through which this occurs, it has been demonstrated that the frequency of reactivation correlates with the severity of the primary infection. When reactivation occurs, progeny virions are produced within the ganglion and travel back down the neuronal axon to be released into the epithelium at or near the site of initial infection. Such reactivation events, which can be either symptomatic or asymptomatic, provide the virus with the opportunity to spread to other susceptible individuals. Interestingly, HSV-1 is more likely to reactivate within a trigeminal ganglion than a sacral ganglion, while the opposite is true for HSV-2. In this way, the tissue tropism of each virus appears to be coupled with a site-specific frequency of reactivation.

Drugs and vaccines

A number of nucleoside analogues have been used effectively to treat herpes infections, including acyclovir (ACV), famciclovir, and valacyclovir. These drugs exploit the fact that viral enzymes recognize certain molecules that the endogenous cellular enzymes do not, enabling the drugs to target only virus-infected cells. One of the most successful antiviral drugs, ACV, a guanine base attached to an acyclic sugar-like molecule, is used to block HSV lytic replication (**Figure 4**). ACV is highly specific because it targets two viral enzymes, virus-encoded thymidine kinase (TK) and DNA polymerase. The viral TK phosphorylates ACV to the monophosphate form while the cellular enzymes phosphorylate it to the di- and triphosphate forms. The HSV DNA polymerase then incorporates the monophosphate form of ACV into the growing DNA chain, but the nascent DNA chain cannot be extended because ACV lacks a 3′-hydroxyl group. Viral DNA synthesis is thereby inhibited. Minimal toxicity is observed because uninfected host cells lack the two enzymes needed for ACV incorporation into DNA.

ACV effectively blocks productive infection but does not affect latent infection. Resistance to ACV is uncommon except in individuals who are immunocompromised, such as AIDS patients, and those undergoing immunosuppression. In such cases, viral replication occurs at a high level and mutant viruses resistant to ACV can arise.

Drugs such as ACV, when given promptly, have proved effective in reducing the mortality and morbidity associated with HSV encephalitis and ocular and neonatal

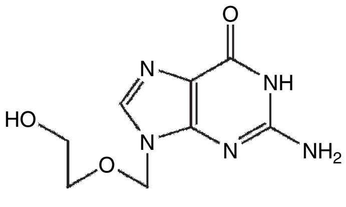

Figure 4 The chemical structure of acyclovir.

infections. However, given the emerging link between HSV-2 and the acquisition and progression of HIV, a method of preventing HSV infection is needed. To this end, a number of different vaccine approaches have been investigated, including killed virus vaccines, subunit vaccines, and genetically engineered live virus vaccines. On the whole, killed virus vaccines have proven ineffective at preventing acquisition of HSV, and of two subunit vaccines that have been through human trials, only one showed some efficacy in a subgroup of patients. At this point, the use of replication-defective mutant viruses as vaccines appears to be the most promising approach. A current candidate, *dl*-529, has been rendered replication-defective through deletions in both U_L5 and U_L29 and has been shown to induce both high titers of neutralizing antibodies and strong cell-mediated responses.

VZV

Disease

VZV is the causative agent of varicella or 'chickenpox', a common childhood disease with the highest prevalence occurring in the 4–10 years age group, and herpes zoster or shingles, a disease most often seen in older individuals. The virus is highly communicable, thought to enter susceptible individuals via the respiratory tract. From here, it spreads to the lymphoid system before producing the characteristic vesicular rash in the skin 10–21 days later. A fever and general feeling of malaise accompany the appearance of the rash. Most individuals become infected with VZV as children. However, approximately 10% of young adults remain susceptible. It should be noted that the incidence of varicella in the United States, particularly in children aged 1–4 years, has declined by approximately 85% since the introduction of a vaccine into the United States pediatric immunization schedule in 1995.

Following a primary infection, VZV establishes a latent infection in the sensory ganglia, with 10–20% of people experiencing a recurrent infection after several decades. Recurrent VZV infection manifests as a vesicular rash in the dermatome innervated by the latently infected ganglion and is referred to as herpes zoster or 'shingles'. It first presents with a prodrome, a painful burning sensation throughout the soon-to-be-affected dermatome. Several days later, the characteristic vesicular rash appears in the skin, which is generally accompanied by flu-like symptoms and can last up to 7–10 days. It is common for the burning, piercing pain to continue after the resolution of lesions in the skin, and in cases where pain persists for longer than 30 days after such resolution the patient is deemed to have post-herpetic neuralgia (PHN). PHN is generally self-limiting and most patients are pain-free by 6 months post-zoster, although pain can be significant until resolution.

The fact that herpes zoster is more common among the elderly and immunocompromised individuals supports the idea that the immune response plays an important role in determining whether VZV is able to reactivate from latency or not and whether symptomatic disease ensues. For those aged 20–50 years, the incidence of herpes zoster is around 0.25%, this rises to 0.8% in people aged over 60 and can reach as high as 50% in people who reach 85 years of age. Individuals with compromised immune systems are also at an increased risk of herpes zoster. People with HIV, leukemia, Hodgkin's and non-Hodgkin's lymphoma, and those who have undergone either bone marrow or renal transplants are at risk. It should also be noted that in immunocompromised individuals, the cutaneous rash is usually more extensive and widespread viremia is also common.

Virus and biology

VZV shares the common herpesvirus virion structure of a core, containing a single copy of the 125 kbp linear, double-stranded DNA genome, surrounded by a nucleocapsid, a proteinaceous tegument layer, and an outer envelope. It also shares the ability to establish lifelong latent infections in the sensory ganglia with the other human alphaherpesviruses HSV-1 and HSV-2. However, unlike the HSVs that are able to replicate in cells from a wide range of hosts, VZV has a very narrow host range, restricted to selected cell types of human and simian origin. Another difference between VZV and HSV relates to the spread of the virus through the host. HSV is generally confined to the epithelium infected during lytic infection and the neuronal cells within the sensory ganglia, where latency is established. VZV on the contrary has the ability to disseminate widely through the bloodstream of the infected host, infecting skin, mucous membranes, and visceral and nervous system tissues.

Replication

The infectious cycle of VZV is similar to that seen with HSV-1 and HSV-2. The virus uses surface glycoproteins, definitely gB and possibly gC, to attach to cellular surface glycosaminoglycans, such as heparan sulfate. Several other viral glycoproteins, gH, gE, and gI are also involved in the attachment and penetration process, following which the viral nucleocapsid, along with several tegument proteins, is transported to the nuclear surface and the viral genome is inserted into the nucleus. It is thought that, like VP16 during HSV infection, the VZV tegument proteins may be involved in initiating transcription of viral genes. The transcription of viral genes occurs in a sequential pattern with IE, E, and L genes, designated as such by the timing of their transcription. Each class of genes is transcribed in the nucleus, the mRNAs are then transported into the cytoplasm where they are translated, and the resulting proteins are then transported back into the nucleus. The IE genes are involved in the regulation of transcription of IE, E, and L genes and each VZV IE protein has homology to one of the HSV IE proteins, although the exact roles played by these proteins are not always homologous. The E proteins are involved in viral DNA replication, and the L proteins are generally structural, used to produce the capsids for progeny virions. The newly formed capsids travel out of the nucleus, becoming enveloped in the process, and are then transported to the cytoplasmic membrane where they are released from the infected cell. In cell culture systems, the entire process can occur in as little time as 8–16 h.

Pathogenesis

VZV is transmitted via inhalation of infectious respiratory secretions or skin-to-skin contact with infectious vesicular fluid and manifests as a vesicular rash throughout the skin 10–21 days later. Due to a lack of animal models for VZV, the pathogenesis of this virus was originally modeled on that of mousepox. Based on this model, it is thought that the inhalation of the virus enables infection of regional lymph nodes, resulting in a primary viremia that enables the virus to disseminate throughout the body, spreading to reticuloendothelial organs such as the liver. Within these organs, it is hypothesized that a phase of viral amplification occurs followed by a second round of viremia during which the virus is transported to the skin.

During primary infection, VZV is able to establish latency within sensory ganglia; VZV DNA is widely detected in the trigeminal ganglia and in many dorsal root ganglia of infected individuals. It is widely accepted that neuronal cells are the primary reservoir of latent virus, infected either hematogenously or by virus transported along neuronal axons from the skin via anterograde axonal transport. VZV latency differs from the situation during HSV infection in two main ways; first, unlike HSV infection, a number of VZV genes are believed to be transcribed and translated while the virus is in a latent state, and second, VZV latency lacks the frequent episodes of asymptomatic reactivation seen with HSV infection. This later observation is thought to be due to the ability of the host immune response to quickly control reactivation events and maintain the virus in a latent state.

Many aspects of the host immune response come into play when dealing with a VZV infection. The innate response in epidermal cells plays an important role in slowing the spread of virus through the skin. NK cells and interferon (IFN-γ) also play a role in the innate defense against VZV infection, helping to contain the virus prior to the development of the adaptive response. In terms of adaptive immunity, VZV induces both cell-mediated and humoral immunity, with T cells thought to be particularly important in clearing infectious virus and helping to maintain VZV in a latent state.

Drugs and vaccines

As with HSV, nucleoside analogues such as ACV and related drugs have been useful in treating VZV infections. In cases of varicella, oral ACV can be given to healthy individuals to reduce the severity of symptoms, while intravenous ACV is given to immunocompromised individuals to reduce the risk of disseminated infections. In cases of herpes zoster, ACV can be given to immunocompetent individuals to shorten the period of lesion outbreaks, the healing period, and the severity of acute neuropathic pain. Similarly for immunocompetent individuals, intravenous ACV can be used to shorten the period of disease and prevent disseminated infection.

VZV is the first HHV for which there is a licensed vaccine, a live attenuated virus vaccine derived from a clinical viral isolate known as the Oka strain. The Oka strain was attenuated by passage into guinea pig fibroblasts, producing a virus with reduced efficiency for replication in human skin. In clinical trials, a single dose of the vaccine was sufficient to induce seroconversion rates of 90% or greater in children 12 years and under and provided complete protection from disease in approximately 85% of exposures. The vaccine induces strong T cell responses with a single dose and also achieves high antibody titers when a second dose is administered and is now recommended for routine vaccination of infants and susceptible older children and adults in the United States. A recent study has also demonstrated that vaccination of healthy adults aged 60 years and older can significantly reduce the frequency and morbidity of herpes zoster, suggesting that a vaccination regimen in this population may also be useful.

The *Gammaherpesvirinae* Subfamily

Gammaherpesviruses are classified as such based on their ability to replicate in epithelial cells, establish latency in lymphocytes, and their oncogenic effects. Within the gammaherpesvirus subfamily, there are two genera: the *Lymphocryptovirus* (LCV) genus, which includes the human pathogen EBV, and the *Rhadinovirus* (RDV) genus, which includes KSHV. It is thought that the viruses within the LCV genus likely evolved from those in the RDV genus.

EBV

Disease

EBV, also known as HHV-4, is a widespread human pathogen with 90% of adults testing seropositive. In developing countries, most children are infected within the first three years of life, while in developed countries around 50% of individuals remain seronegative through childhood. It is estimated that 25% of people who then acquire EBV during adolescence or young adulthood will present with acute disease called infectious mononucleosis (IM), although it should be noted that childhood cases of IM are common in Asian populations and are possibly underdiagnosed in other parts of the world. Patients with IM can present with symptoms ranging from a mild and transient fever to a period of malaise and pharyngitis lasting several weeks. This period of EBV disease is closely linked to the emergence of the cytotoxic T cell response to infection, and it is widely accepted that IM is mostly an immunopathologic disease, with the proinflammatory cytokines secreted by active T cells thought to be responsible for many of the IM symptoms. In a small subset of patients, IM is poorly controlled and can lead to more severe, possibly fatal, outcomes. Males with X-linked lymphoproliferative (XLP) syndrome are highly sensitive to EBV infection. In these patients, primary EBV infection results in severe IM-like symptoms and can rapidly result in mortality, thought to be due to an uncontrolled T cell response to infection. Virus-associated hemophagocytic syndrome (VAHS) and chronic active EBV infection (CAEBV) are two other serious outcomes of EBV primary infection. Both involve EBV infection of T cells, resulting in virus-driven proliferation of these cells (much like the proliferation of B cells during a classical primary infection). The infected T cells release huge amounts of proinflammatory cytokines, which results in hemophagocytosis, where macrophage begin phagocytosing red blood cells, platelets, leukocytes, and other cells. The risk of mortality with either syndrome is high.

Following primary infection, EBV establishes a latent infection in B cells, which is generally maintained as such for the life of the host with no clinical manifestations. However, this is not always the case, and EBV is associated with a number of different human malignancies. In immunocompetent individuals, usually following several decades of EBV latency, EBV is associated with the development of certain types of Hodgkin's lymphoma, several B-lymphoproliferative lesions, T cell and NK cell nasal lymphomas, and gastric and nasopharyngeal carcinomas. In immunocompromised individuals, the virus is capable of rapid tumor development, with some cases of tumorigenesis evident within months of EBV infection. In transplant settings, a link has been demonstrated between EBV and posttransplant lymphoproliferative disease (PTLD), with EBV association as high as 100% in early onset cases and 80% with late onset cases. AIDS patients show a heightened risk of B cell lymphoma with approximately 50% linked to EBV, and most settings involving immunosuppression have demonstrated a link between EBV and smooth muscle cell tumors. EBV is also associated with endemic Burkitt's lymphoma (BL), the most common childhood cancer in equatorial Africa which is geographically linked to areas of holoendemic malarial infection, although the mechanisms through which EBV contributes to BL are not fully understood.

Virus and biology

EBV shares the common herpesvirus virion structure and has a 184 kbp genome. There are two strains of the virus, types 1 and 2 or types A and B, which circulate in most populations. Individuals can be infected with both types and this is a common occurrence in immunocompromised individuals. Type 1 is generally more prevalent in developed countries while type 2 is dominant in equatorial Africa and New Guinea. The main differences between the two strains are seen in the nuclear protein genes that encode EBV-coded nuclear antigen (EBNA)-LP, EBNA-2, EBNA-3A, EBNA-3B, and EBNA-3C.

Replication

EBV is known to infect both B cells and epithelial cells during primary infection, with infection of epithelial cells thought to result in a lytic infection and infection of B cells thought to generally result in a latent infection. In B cells, the virus binds to CD21 and MHC class II on the surface of target cells using glycoproteins in the virus envelope. A cell surface receptor for epithelial cell infection has yet to be found, and it is hypothesized that virus may be transferred to epithelial cells directly from lytically infected B cells. The entry of virus into each cell type differs in that virus enters B cells via the endocytic pathway while virus entering epithelial cells does so at the cell surface, in both cases viral glycoproteins are involved in facilitating fusion and entry of the virus into the target cell. Following entry of the virus into the target cell, the nucleocapsid is transported to the nucleus into which the viral genome is inserted.

If a lytic infection ensues, as is thought to occur in epithelial cells *in vivo*, the sequential expression of IE, E, and L genes, common to herpesviruses, is seen. The IE proteins are primarily involved in activation of E gene expression, the E proteins are primarily involved in viral DNA replication, while the L proteins are involed in the production of progeny virions. At this time, it is thought that newly formed nucleocapsids initially acquire an envelope at the inner nuclear membrane, are de-enveloped as they are released into the cytoplasm, and then reacquire an envelope as they bud through the plasma membrane.

It is widely accepted that initial infection of B cells results in a latent or persistent infection. In fact, *in vitro* studies demonstrated that infection of B cells with EBV resulted in a latent infection that was capable of causing perpetual B cell proliferation, helping to confirm the oncogenic properties of EBV. During latency, the viral genome is maintained in the nucleus of the infected cell in an episomal form with variable levels of gene expression possible. There are four different forms of EBV latency: latency 0, I, II, and III. These stages are classified as such based on the level of expression of the EBNAs and latent membrane proteins (LMPs), with latency 0 having no viral antigens expressed and latency III having all latency proteins expressed.

These different forms of latency are used by the virus to ensure the maintenance of the viral genome within progeny B cells, and as such the virus uses cellular differentiation controls to determine which level of latency is required at any given time. For example, during a primary infection, EBV is present in infected B cells in latency III form, and the expression of viral genes at this stage is used to drive the proliferation and differentiation of these cells into a latently infected memory pool. Once the memory pool is established, and in order to prevent further detection by virus-specific cytotoxic T cells, EBV switches off all gene expression, thus entering latency 0. At times when memory B cells are induced to divide by homeostatic signals, the virus reactivates to latency I to ensure the viral genome is not lost during such cell division. In this way, the virus is able to establish a balance between avoiding immune detection and maintaining the viral genome. At times of cell proliferation, the viral EBNA-1 protein tethers the viral chromosome to the cellular chromosome to ensure maintenance of the viral genome within daughter cells.

Pathogenesis

EBV infection is transmitted via the oral route and is generally asymptomatic. As such, knowledge of the primary infection has come from the study of IM. These individuals shed virus in saliva and throat washings. However, the source of this virus remains contested. It is generally assumed that because B cell-deficient individuals show no sign of EBV infection in the throat, initial infection of a naïve host is B cell-dependent. However, it is becoming more widely accepted that epithelial cells may also be sites of viral replication, with studies suggesting that virus bound to the surface of a B cell is highly efficient at infecting epithelial cells. Interestingly, recent evidence further suggests that virus released from B cells is defective for B cell infectivity but shows enhanced infection of epithelial cells, while virus released from epithelial cells has the opposite phenotype.

EBV establishes a latent infection in B cells at the site of primary infection, the tonsillar tissue. Studies suggest that latently infected B cells express a memory phenotype, and it has been proposed that infection of naïve B cells with EBV mimics the process of B cell differentiation, resulting in activation and proliferation of the infected cell population, and thus the production of an expanded pool of latently infected memory cells. It is generally accepted that the cell-mediated immune response brings the proliferating B cells under control. The memory pool of latently infected B cells, which circulates through the body, is able to disperse the latently infected cells throughout the lymphoid system. Individuals latently infected with EBV will have peripheral blood B cells that harbor virus and, following clearance of the primary infection, these individuals will continue to shed low levels of infectious virus via the oral cavity. This virus comes from the latent B cell reservoir. It is thought that memory B cells containing latent virus may undergo reactivation when they receive an activation signal, and that such cells, which localize near mucosal surfaces, would be capable of transmitting lytic virus to epithelial cells where viral replication and subsequent shedding can occur.

In a healthy individual with an intact immune system, EBV persists in this form for the life of the host with no clinical manifestations. The latent pool is constantly maintained by the virus moving forward and backward through the various forms of latency as required, and infectious virus is sporadically shed from the oral cavity with the potential of infecting other susceptible hosts. However, when immune suppression occurs, either through disease or drug intervention, this balance is destroyed and the individual is put at risk of EBV-associated disease.

Drugs and vaccines

To date, attempts to develop preventative vaccines against EBV have been largely unsuccessful. The finding that the major viral envelope protein gp350 was the dominant target of the neutralizing antibody response led to attempts to develop a gp350 subunit vaccine. However, some evidence suggests that neutralizing antibodies alone are not sufficient to protect against EBV, with clinical trials showing that the gp350 subunit vaccine failed to prevent primary infection, although it was able to reduce the incidence of IM symptoms. It is now widely believed that an integrative approach is required, where a vaccine to prime the antibody response (such as the gp350 subunit vaccine) would be given in combination with a vaccine aimed at priming the $CD8^+$ T cell response. However, it should be noted that even this integrative approach would probably be most successful at limiting rather than preventing infection.

In parallel with efforts to design a preventative vaccine, efforts are also being aimed at developing immunotherapeutics for EBV-associated malignancies. Current strategies being developed include adoptive transfer of activated T cells specific for viral antigens expressed on EBV-associated tumors and the development of vaccines that can boost the host T cell response to these same antigens. Both strategies are aimed at increasing T cell recognition and subsequent destruction of EBV-associated tumors.

KSHV

Disease

KSHV, also known as HHV-8, is the most recently identified HHV. The virus was originally identified because of its association with Kaposi's sarcoma (KS), an endothelial neoplasm. It was later recognized as a member of the LCV genus within the gammaherpesvirus subfamily of herpesviruses.

Infection rates with KSHV in the United States and in Europe are relatively low with approximately 3% of the population infected. In Africa, a different picture emerges, with KSHV reaching endemic rates of infection of between 40 and 60%. The various strains of KSHV can be divided into four major groups or clades: A, B, C, and D, with each virus within a given clade sharing a single common ancestor. A and C tend to cluster together and are more prevalent in Europe and in the United States, B is the most commonly isolated in infected individuals in sub-Saharan Africa, and D is the dominant clade seen in South Asia and Australia. The pattern of distribution and the evolutionary relationship between viruses in the different clades suggest that KSHV entered the human population at about the time when modern man emerged in Africa, with the different clades being produced as different groups moved out of Africa to Europe and Asia, respectively. The fact that the distribution of these clades seems to have been maintained over millions of years also suggests that, particularly in areas of high seroprevalence, transmission of KSHV is primarily familial, moving vertically from parent to child and horizontally between different members in a family unit possibly via salivary exchange. It should be noted that in areas of low seroprevalence, such as Europe and the United States, the pattern of infection appears to follow that of a sexually transmitted disease, and virus has been successfully isolated from both saliva and genital secretions.

The most common malignancy associated with KSHV is KS. KS is a complex, angioproliferative, and inflammatory lesion. It was historically a disease of elderly Mediterranean men until it emerged as the most common neoplasm seen as a complication of HIV/AIDS. In both settings, the disease is a slow progressing malignancy, although it can result in death in AIDS patients if organ involvement is present. Unlike a classical tumor, KS lesions contain many different cell types with the driving cell being a KSHV-infected spindle cell (an elongated endothelial cell). The spindle cells produce proinflammatory and angiogenic products and may actually require factors released from proinflammatory cells for survival and growth. It is possible for KS lesions to be locally or systemically invasive, requiring chemotherapy or radiotherapy.

While strong evidence suggests that KSHV is necessary for KS development, it is certainly not sufficient. Within the general population, only 1 in 10 000 infected individuals will develop KS annually. Therefore, it is assumed that there are other cofactors involved in the development of KS. In AIDS-related KS, the assumption is that HIV infection is the cofactor. It has been proposed that an HIV protein may act as a growth factor for KSHV or that the immunodeficiency seen during HIV infection may enable KSHV to disseminate more widely through the host, increasing the chances of endothelial cell infection. The cofactor in non-AIDS-related KS remains unknown.

Along with KS, KSHV has been implicated in two B cell diseases, primary effusion lymphoma (PEL) and Castleman's disease. PEL is a rare disease seen in end-stage AIDS patients and is characterized by proliferation of B cells primarily in body cavities such as the pleura, pericardium, and peritoneum. Unlike KS, PEL is a classical malignancy with every cell in the tumor harboring KSHV DNA. Castleman's disease is a rare, lymphoproliferative lesion that is seen in both HIV-positive and HIV-negative individuals. In HIV-negative individuals, Castleman's disease generally presents as a benign tumor localized to a single lymph node. This form of Castleman's disease does not involve KSHV and is usually treated by excision of the involved tissue. Multicentric Castleman's disease is a more aggressive, systemic illness characterized by sustained fever, sweats, and weight loss. This form of Castleman's disease is seen with increased frequency in patients with AIDS and, in this setting, is almost always linked to KSHV infection.

Virus and biology

KSHV shares the standard herpesvirus virion structure described above. The virion contains a double-stranded linear DNA genome of between 165 and 170 kbp in length that contains four blocks of highly conserved genes, many of which encode replication proteins common to alpha- and betaherpesviruses. The genome also encodes several small noncoding mRNAs, the function of which remains unknown, several of which are expressed during latency and one of which is expressed during lytic infection.

Replication

The replication cycle of KSHV follows a pattern similar to that seen with the other herpesviruses and thus will not be discussed in detail here. Virus attachment and entry are facilitated by several viral glycoproteins, following which the viral genome and several tegument proteins are delivered into the nucleus of the infected cell. If, at this point, the virus enters the lytic cycle, the sequential expression of over 90 viral genes is initiated. These genes are divided into IE, delayed early (DE), and L genes. The mechanism of KSHV egress has yet to be fully elucidated.

Despite the obvious ability of the virus to induce a lytic infection, as demonstrated by the intermittent shedding of virus from infected individuals, studies in cell culture systems suggest that the default pathway in KSHV infection is latency. From *in vitro* work, it appears that only a small number of cells (1–3%) will enter the true lytic pathway and that this will subside following several days of infection. In the majority of cells, a defective version of lytic infection arises, which is quickly terminated and latency established. In these cells, a range of lytic cycle genes are expressed during the first 12 h of infection. However, the expression of these genes ceases by 24 h postinfection and the genes are not expressed in their correct sequential order, thus providing evidence of the defective nature of this 'lytic' infection. Latency is then quickly established in these cells. The role that the faulty lytic infection plays in the overall virus infection remains unknown, although it has been proposed that the transient expression of some of the viral immune evasion mediators may be beneficial during the early stages of infection.

Once latency is established, the viral genome is replicated as an episome. The viral LANA protein tethers the viral episome to the cellular chromosome so that the viral genome is distributed to progeny cells during cell division. At this time, only a few of the 90-plus viral genes are expressed. However, the exact roles that these proteins play during the latent infection still require much investigation. Work to date would suggest that they may be involved in maintenance of the viral genome in dividing cells, prevention of apoptosis and, surprisingly, upregulation of proinflammatory responses. The switch from the latent to the lytic phase of infection is thought to be facilitated by the so-called lytic switch protein, known as RTA. This protein is a viral transcriptional activator that is capable of inducing lytic gene expression on its own, but becomes even more efficient when bound to one of several different HCFs. In experimental systems, deletion of RTA prevents both spontaneous and chemically triggered induction of the lytic cycle.

Pathogenesis

Primary infection of a susceptible host is followed quickly by the establishment of latency, primarily in B cells. In most individuals, the latent infection is asymptomatic and is accompanied by intermittent, clinically silent viral reactivation that enables shedding of virus in the saliva. Given the apparent absence of an extended primary lytic infection, asymptomatic reactivation during latency would appear to play a major role in virus transmission.

The exact role of KSHV in the pathogenesis of KS is currently unknown. It appears that both latent and lytic stages of infection are important, with KSHV latency much less potent than EBV latency at inducing cell immortalization. Most KS spindle cells are latently infected but a small percentage demonstrates lytic infection. It would appear that this low level of lytic replication is important in the development of KS, possibly enabling the reinfection of spindle cells that have lost the KSHV genome, providing a reservoir of newly infected cells to replace cells within the tumor mass that have died, or even providing some of the inflammatory and angiogenic signals that play a role in KS pathogenesis.

Drugs and vaccines

There are currently no drugs or vaccines available for the prevention or treatment of KSHV. In most individuals, the immune response is adequate to control the virus and prevent virus-associated disease. With this in mind, in immunocompromised individuals, it is common to treat the underlying cause of immunosuppression or to attempt to treat the malignancy itself rather than the viral infection.

The *Betaherpesvirinae* Subfamily

The betaherpesviruses are characterized by a restricted host range, a long productive cycle, and the ability to establish latent infections in secretory glands, lymphoreticular cells, and kidneys. The betaherpesviruses have the highest level of evolutionary and genetic diversity of the three herpesvirus subfamilies, which can make the use of animal models to study human pathogens within this subfamily difficult. There are four genera within the betaherpesvirus subfamily: the cytomegaloviruses, the muromegaloviruses, the roseoloviruses, and the proboscivirus (which has only a single member).

HCMV

Disease

HCMV is a ubiquitous human pathogen, infecting those in developing countries in their youth and those in developed countries across a slightly wider timeframe. In most individuals, HCMV causes an asymptomatic infection, with disease generally only seen in those unable to mount a cellular response to infection, such as neonates or individuals with some form of immunosuppression. As is characteristic of herpesviruses, HCMV establishes a latent infection within the host, although interestingly this is also accompanied by what can be called a chronic infection, with infected individuals shedding virus sporadically from their bodily fluids for life.

HCMV can be classified as an opportunistic pathogen, only causing disease in situations where the immune response is severely compromised (such as HIV) or absent (such as congenital infection). In most healthy individuals, infection with HCMV is clinically silent, although it should be noted that in a small number of cases a short bout of fever and malaise can occur, similar to the mononucleosis caused by EBV.

Congenital infection

Transmission of HCMV from mother to fetus or newborn is a very common occurrence and can occur via three routes: transplacental, intrapartum, and via human milk. HCMV is the only herpesvirus known to exhibit natural transplacental transmission, and it is this congenital route of transmission that causes serious morbidity. That said, intrapartum and transmission via breast milk, while not associated with the morbidity of congenital infections, both play an important role in viral epidemiology. These newborn children infected with HCMV will continue to shed virus capable of infecting susceptible hosts for many years after the primary infection.

Congenital infection can occur when the mother has either a primary or reactivated infection during pregnancy, with evidence from those undergoing a primary infection suggesting that transmission to the fetus can occur in 20–40% of cases. Although less than 1% of live births involve a child with congenital HCMV, the long-term sequelae for these children make it a serious disease, with HCMV estimated to be the leading cause of infectious brain damage in the United States. Approximately 5–10% of those born with a congenital HCMV infection will be symptomatic, showing clinical manifestations such as hearing loss, seizures, jaundice and brain abnormalities, with long-term sequelae such as mental retardation, cerebral palsy, and impaired vision. In 10% of cases, symptomatic congenital HCMV infection will be fatal. Even the 90% of congenitally infected children born without symptoms remain at risk of long-term CNS sequelae such as hearing loss.

Infection in an immunocompromised host

Patients with compromised or suppressed immune systems are at greater risk of CMV-associated disease than healthy individuals, with the severity of disease often matching the level of immunosuppression. In patients with HIV or those undergoing solid organ or hematopoietic stem cell transplants, HCMV can disseminate into a number of different organs, causing clinical manifestations such as pneumonitis, retinitis, and hepatitis. In organ transplant patients, HCMV has also been shown to cause dysfunction of the transplanted organ and put the patient at greater risk of fungal and bacterial infections. The thorough screening of transplant patients and the use of antivirals in this setting and HAART in the HIV setting is helping to reduce the morbidity and mortality associated with HCMV infection in immunocompromised individuals.

Virus and biology

Compared to the other HHVs, HCMV is a very large virus. With a genome between 196 and 241 kbp, the virus encodes in excess of 166 gene products (less than half of which are conserved in all betaherpesviruses), and while HCMV shares the common herpesvirus virion structure, the actual size of the virions is larger than that of the other HHVs.

Replication

The replication cycle of HCMV follows a similar pattern to that described for the other herpesviruses. The virus uses heparan sulfate as an initial binding receptor on target cells and then enters the cell via either fusion of the viral envelope with the cellular membrane or the endocytic pathway. The viral genome is released into the nucleus and the lytic viral genes are expressed in a sequential manner: IE, DE, and L. Unlike the alphaherpesviruses, evidence suggests that HCMV most likely undergoes a two-stage envelopment/de-envelopment/re-envelopment process in order to exit an infected cell. Newly synthesized nucleocapsids are enveloped as they pass through the inner nuclear membrane and then deenveloped as they pass through the outer nuclear membrane. The envelope-free nucleocapsid is thus released into the cytoplasm where it reacquires an envelope at the ERGIC membranes before being transported out of the infected cell via the cellular exocytic pathway.

In terms of replication, the main difference between HCMV and other HHVs is the length of the replication cycle. DE gene expression begins at 6 h postinfection and continues through 18–24 h when viral DNA synthesis is initiated. From initial attachment to the initiation of progeny virion release, the complete infectious cycle takes between 42 and 78 h. During this time, the virus has a profound effect on the infected cell, blocking IRF-3 activation, IFN signaling, and apoptosis responses and interrupting the cell cycle in such a way that infected cells are able to survive for several days of productive infection.

Pathogenesis

In an immunocompetent host, HCMV infection is generally asymptomatic. Primary infection is usually initiated in the mucosal epithelium following direct contact with infectious secretions from another individual, aerosol transmission does not occur. A systemic phase of infection then follows with a leukocyte-associated viremia. Animal models with murine CMV (MCMV) suggest that the virus uses immature leukocytes from the bone marrow to facilitate dissemination to the salivary glands, kidneys, and other tissues. This systemic phase of infection is associated with high levels of persistent viral shedding in the saliva, urine, breast milk, and genital secretions and continues for a long time after the onset of the adaptive immune response. It can last for months in adults and for years in young children, supposedly due to a less effective cellular immune response in younger patients. The ability of the virus to persist in the face of the cellular immune response is thought to be due, in part, to the fact that more than 25 viral genes have been found to play a role in modulating the host response to infection.

When virus is cleared following primary infection, HCMV is maintained in a latent state in hematopoietic progenitor cells (HPC). However, unlike the human alphaherpesviruses, this latent infection is accompanied by what can be called a chronic infection in epithelial cells of the salivary glands and kidneys, which results in sporadic shedding of virus in the bodily fluids for the life of the host. Reactivation of virus from latency, as opposed to the sporadic viral shedding achieved by the persistent infection in the salivary glands and kidneys, seems to be an issue only in situations of immunosuppression.

Immune responses to HCMV are well maintained for years beyond the primary infection, at levels not seen with other herpesviruses or persistent infections. Innate immune responses such as IFN and NK cells are important during the early stages on infection and may play a role in containing the infection until the adaptive immune response develops. T cell responses appear to be of greater importance than antibody responses, although in certain settings antibodies play a crucial protective role. Despite the persistence of the primary viremia in the face of an active cellular immune response, the fact that viral reactivation is only seen in cases of immunosuppression suggests that the immune response plays an important role in helping to maintain the virus in a latent state and preventing CMV-associated disease.

Drugs and vaccines

There are currently four drugs approved for the treatment of HCMV infection in immunocompromised individuals: Ganciclovir, Valganciclovir, Foscarnet, and Cidofovir. All four have been shown to reduce or eliminate viremia, reduce viral shedding, and prevent or control CMV disease. However, due to risks of severe toxicity, the drugs are only used when a patient is at risk of serious disease. At this time, no drugs are approved for the treatment of congenital CMV, although a small Ganciclovir trial did produce some positive results.

Given the seriousness of congenital CMV infection and the difficulty in preventing maternal infections, a preventative vaccine would have a large public health benefit. Several different vaccine approaches have been tested to date. However, a CMV vaccine is yet to reach the market. Strategies that have reached clinical trials have included a live attenuated vaccine, a gB subunit vaccine, a canary pox vector expressing CMV gB and pp65, and a DNA vaccine with antibody and CTL epitopes. Several of these vaccines have shown promising results in inducing strong immune responses.

HHV-6 and HHV-7

Disease

HHV-6 was first isolated in 1986 and is classified into two variants, A and B. HHV-6B is the major causative agent of exanthem subitum (ES), while HHV-6A has not been clearly linked with any disease. HHV 7 was first isolated in 1990 and is also a causative agent of ES as well as being associated with febrile convulsions in young children. Both HHV-6 and HHV-7 are ubiquitous pathogens, with greater than 90% of adults seropositive for both.

ES or roseola is a classical childhood disease (sixth disease). It initially presents as a fever lasting for 3–4 days. As the fever clears, a rash appears, first on the trunk and hands and then on the lower limbs, lasting several days. HHV-6B is the major cause of ES, with the magnitude of viral replication correlating with the severity of disease. HHV-7, while also a cause of ES, has a lower frequency of disease as compared to HHV-6B. It is possible for children to have successive bouts of ES caused by one virus and then the other. Most cases of ES are benign and are associated with other symptoms such as diarrhea, cough, and febrile convulsions. However, it is possible for HHV-6 infection to result in encephalitis, meningitis, and hepatitis, which can be fatal.

Following primary infection, both HHV-6 and HHV-7 persist in a latent state. As with other herpesviruses, reactivation is generally only a problem in situations where the immune system is compromised. In bone marrow transplant recipients, asymptomatic HHV-6 reactivation is common. However, reactivation has also been linked to bone marrow suppression, encephalitis, colitis, pneumonitis, and graft-versus-host disease. In solid organ transplant recipients, HHV-6 reactivation has been associated with kidney rejection.

Virus and biology

HHV-6A, HHV-6B, and HHV-7 are members of the roseolovirus genus of the betaherpesvirus family, sharing the common characteristics of growth in T cells, high prevalence, and association with febrile rash illness. HHV-6A and HHV-6B are closely related but actually meet the requirements for recognition as two separate viruses – they differ in cell tropisms, interactions with cells and the immune system, DNA sequences, and epidemiology. As with the other human betaherpesvirus, HCMV, both HHV-6 and HHV-7 have protracted replication cycles and share some beta-subfamily-specific genes.

The virion structure of HHV-6 and HHV-7 follows the common herpesvirus structure of a dsDNA genome within an icosahedral capsid that is surrounded by a tegument layer and finally a lipid bilayer envelope.

HHV-6 has a genome of up to 170 kbp, while the HHV-7 genome is 145 kbp in length.

Replication

HHV-6 and HHV-7 have a similar replication cycle to that of the other HHV. Following virus attachment and entry, the viral genome is delivered to the nucleus where viral gene transcription is initiated in a sequential manner: IE, E, and L. Egress of newly formed virions from the infected cell follows the same path as that used by HCMV: envelopment/de-envelopment/re-envelopment.

Pathogenesis

Transmission of HHV-6 and HHV-7 is not fully understood. It is thought that transmission during infancy occurs horizontally, possibly via saliva during close personal contact. However, it is also thought that the viruses can be transmitted across the placenta, during delivery, or even intrauterine. The exact site of primary infection is also yet to be determined. It is currently thought that infection is initiated through respiratory pathways, however the exact cells that are infected are not known.

Both HHV-6 and HHV-7 establish latent infections within their hosts. It is thought that HHV-6 establishes latency in monocyte or macrophage cells and certain stem cells. HHV-6 DNA and antigens can also be detected in a range of other sites including the saliva, brain, and lung, suggesting a concurrent persistent infection. HHV-7 establishes a latent infection in $CD4^+$ T cells while maintaining a persistent infection in the salivary glands and a variety of other tissues.

Drugs and vaccines

Several drugs approved for use against CMV have been shown to be effective against HHV-6 and HHV-7 *in vitro*, these include Ganciclovir, Foscarnet, and Cidofovir. IFN-α and IFN-β have also been shown to inhibit HHV-6 replication *in vitro*. However, no drugs are currently approved for use in the treatment of HHV-6 or HHV-7 infection.

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HIV/AIDS

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Glossary

acquired immunodeficiency syndrome (AIDS) An immune system disease caused by human immunodeficiency virus (HIV) infection that is marked by significant depletion of CD4+ T cells resulting in increased susceptibility to a variety of opportunistic infections, certain cancers, and neurological disorders.

adaptive immunity Host defenses that are mediated by B and T cells following exposure to antigen and that exhibits specificity, diversity, memory, and self–nonself recognition.

CD4 A cellular surface glycoprotein found majorly on T cells that facilitates recognition of the T cell receptor (TCR) to antigens bound to major histocompatibility complex (MHC) class II complexes and serves as a receptor for HIV binding.

chemokines Class of proinflammatory cytokines that mediate, attract, and activate leukocytes.

cytokines Low-molecular weight proteins secreted by leukocytes and some other cells that act as intercellular mediators and regulate the intensity and duration of the immune response.

dendritic cells (DCs) Bone-marrow derived cells that descend through the myeloid and lymphoid lineages and are specialized for antigen presentation to T cells.

humoral immunity Host defenses that protect against extracellular pathogens and are mediated by B cell-secreted antibodies present in the plasma, lymph, and tissue fluids.

innate immunity Nonspecific host defenses that occur rapidly in response to a pathogen and involve anatomic, physiologic, endocytic, phagocytic, and inflammatory mechanisms.

lentivirus A genus of the family Retroviridae consisting of nononcogenic retroviruses that cause diseases characterized by long incubation periods and persistent infection. Lentiviruses are unique as they have open reading frames between the *pol* and *env* genes and in the 3′ *env* region.

long-terminal repeats (LTRs) Several hundred nucleotides long, identical DNA sequences, found at either end of transposons and proviral DNA. LTRs are formed by reverse transcription of retroviral RNA and function as promoters of viral transcription.

macrophage Mononuclear phagocytic leukocytes that play roles in adaptive and innate immunity. There are many types of macrophage present in blood or fixed in tissues.

retroviridae A family of viruses with single-stranded RNA that upon infection generate a DNA copy via a viral reverse transcriptase (RT).

T lymphocyte A lymphocyte that matures in the thymus and circulates in the blood and thymic tissue. It participates in the normal function of the immune system and expresses a TCR, and CD3 and CD4 or CD8.

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ADE	antibody-dependent enhancement
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
ARS	acute retroviral syndrome
ARV	AIDS-associated retroviruses
CAF	cell antiviral factor
cDNA	complementary DNA
CLRs	C-type lectin-like receptors
CMV	cytomegalovirus
CNAR	cell noncytotoxic antiviral response

CRF	circulating recombinant form
CTL	cytotoxic T lymphocyte
DC	dendritic cell
FIV	feline immunodeficiency virus
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
HTLV	human T-cell leukemia virus
KIR	killer cell immunoglobulin-like receptor
LAS	lymphadenopathy syndrome
LAV	lymphadenopathy-associated virus
LC	Langerhans cell
LTNP	long-term nonprogressors
LTR	long-terminal repeat
LTS	long-term survivors
MDC	myeloid dendritic cell
MDDC	monocyte-derived dendritic cell
MHC	major histocompatibility complex
MHR	major homology region
NOD	nucleotide-binding oligomerization domain
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
NSI	nonsyncytia-inducing
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PDC	plasmacytoid dendritic cell
PEP	postexposure prophylaxis
PIC	preintegration complex
PRR	pathogen recognition receptor
RIG-1	retinoic acid-inducible gene 1
RLR	RIG-1-like-receptor
RRE	Rev response element
RT	reverse transcriptase
SIV	simian immunodeficiency virus
SNP	single-nucleotide polymorphism
Tcm	central memory T cells
TCR	T cell receptor
Tem	effector memory T cells
TLR	toll-like receptor
VCAM	Vascular cell adhesion molecule

Defining Statement

HIV pathogenesis and immune system.

Introduction

The human immunodeficiency virus (HIV) is a member of the genus *Lentivirus* in the Retroviridae family, a large and diverse family of enveloped RNA viruses. Retroviruses are so called because their RNA genome is transcribed into linear double-stranded DNA by a characteristic enzyme known as reverse transcriptase (RT), a RNA-dependent DNA polymerase that reverses the classical flow of genetic information. The DNA subsequently enters the nucleus and integrates as a DNA provirus into the host cellular genome. The integrated retrovirus then is either transcriptionally active producing virions or remains in a silent or latent state.

Lentiviruses consist of a diverse group of animal viruses with certain clinical and biological characteristics. The human counterpart, HIV, was discovered because of its association with the AIDS. This clinical condition is characterized by a marked reduction in the numbers of CD4+ T cells and a loss in immune function leading to the development of various opportunistic infections and cancer (**Table 1**).

Table 1 Average CD4+ cell count at diagnosis of an AIDS-defining condition

Opportunistic infection	*CD4+ cell count* μl^{-1}
Tuberculosis	<400
Herpes zoster	<300
Non-Hodgkin's lymphoma	240
Kaposi's sarcoma	220
Pneumocystis jiroveci pneumonia[a]	120
Toxoplasmic encephalitis	98
Cryptococcal meningitis	73
Primary cerebral lymphoma	<50
Mycobacterium avium complex infection	<50
Cytomegalovirus (CMV) retinitis	<50

[a] Formerly *P. carinii*.

Discovery of the AIDS Virus

Recognition of AIDS and HIV-1

After over two decades, HIV and AIDS have continued to challenge public health approaches all over the world. The syndrome was first recognized in the United States in 1981 with the appearance of Kaposi's sarcoma and *Pneumocystis jiroveci* (*carinii*) pneumonia in young men. It was primarily found in homosexual men and intravenous drug users but soon was recognized in infants born of infected mothers (see 'Overview'). Initially, the symptoms characteristic of this disease reflected opportunistic infections and cancers. The underlying cause was then identified as an immune deficiency with a major loss of CD4+ T cells. The syndrome became known as the AIDS and in the early 1980s was found to be present in Europe, the Caribbean, and many parts of Africa.

The first indication that a retrovirus could be the etiologic agent of AIDS came in 1983 when Francoise Barre-Sinoussi and colleagues recovered a virus from the lymph node of a person suffering from lymphadenopathy syndrome (LAS). The isolated virus had the morphology of a budding retrovirus, later recognized as a lentivirus. It had the unusual characteristic of infecting peripheral blood mononuclear cells (PBMCs) and causing cytopathic effects within 6–7 days. This LAS agent termed as lymphadenopathy-associated virus (LAV) did not establish a transformed state in CD4+ cells, but caused cell death after high-level replication.

Subsequently, two other groups identified retroviruses in AIDS patients. In 1984, Robert Gallo and coworkers reported a retrovirus that they called (human T-cell leukemia virus) HTLV-III because they believed it to be a part of the HTLV family of oncogenic viruses. At the same time, other retroviruses were identified in subjects from San Francisco by Jay Levy and colleagues in 1984. They had the characteristics of cytopathic agents like LAV and were called AIDS-associated retroviruses (ARV). These three prototype viruses, LAV, HTLV-III, and ARV, were subsequently recognized as members of the same group of viruses belonging to the genus *Lentivirinae* in the family Retroviridae, distinct from HTLV. In 1986, the International Committee on Taxonomy of Viruses named this group of viruses as HIV. After identification of another type of this virus in West Africa, which was named HIV-2, the earlier type was named HIV-1. Both these viruses bear a sequence homology of about 45% and exhibit heterogeneity in biological behavior.

HIV-2

A second AIDS retrovirus, HIV-2, recovered from AIDS patients from West Africa, particularly the Cape Verde Islands and Senegal, differs in sequence by more than 55% from the earlier isolated HIV-1 strains. Subsequently, other HIV-2 isolates were recovered from individuals from Guinea Bissau, Gambia, and Ivory Coast. The genome of HIV-2 is similar to that of HIV-1 except for the presence of the accessory protein Vpx and the absence of Vpu. The major serologic difference between HIV-2 and HIV-1 isolates resides in the envelope glycoproteins. Antibodies to HIV-2 generally cross-react with Gag and Pol proteins of HIV-1 but do not detect HIV-1 envelope proteins and vice versa. However, HIV-2 envelope glycoproteins appear to cross-react serologically with envelope proteins from isolates of simian immunodeficiency virus (SIV), a group of primate lentiviruses, thus suggesting that HIV-2 was derived from SIV.

The HIV Virion: Structure and Genomic Organization

Overview

Like all retroviruses, the HIV virion is about 100–120 nm in diameter, with heterogenous morphology. The mature infectious virus particle buds from the cell membrane forming a sphere with an outer lipid bilayer consisting of gp120 and gp41 envelope glycoproteins and a dense, cone-shaped core composed of the viral p24 Gag capsid (CA) protein enclosing the two molecules of single-stranded RNA (**Figure 1**). The Gag matrix (MA, p17) protein forms the inner shell below the viral membrane and the nucleocapsid (NC, p7). The Gag protein interacts with the viral RNA inside the capsid. The two RNA strands are also associated with a RNA-dependent DNA polymerase (Pol), also called RT. Several other viral accessory proteins required for the early phases of virus infection are present within the virion: Vif, Nef, and Vpr (as well as Vpx in HIV-2). Certain cytoskeletal proteins (e.g., actin, ezrin, emerin, moesin, and coflin) have also been detected within virions. HIV isolates have demonstrated selective incorporation of specific lipid domains from the host cell membrane during viral budding.

The genome of HIV is about 10 kb with open reading frames coding for three structural (Gag, Pol, and Env) and six accessory proteins (Vif, Vpr, Vpu, Rev, Tat, and Nef). HIV-1 and HIV-2 follow the basic genomic structure common to most retroviruses: *gag–pol–env* structural genes symmetrically flanked by two complete viral long-terminal repeats (LTRs) (**Figure 2**). These LTRs contain transcriptional regulatory sequences, RNA processing signals, and packaging and integrating sites. The primary transcript of HIV is a full-length viral mRNA, which is translated into Gag and Pol proteins. The 55 kDa Gag precursor protein (p55) is synthesized on cytosolic ribosomes and becomes

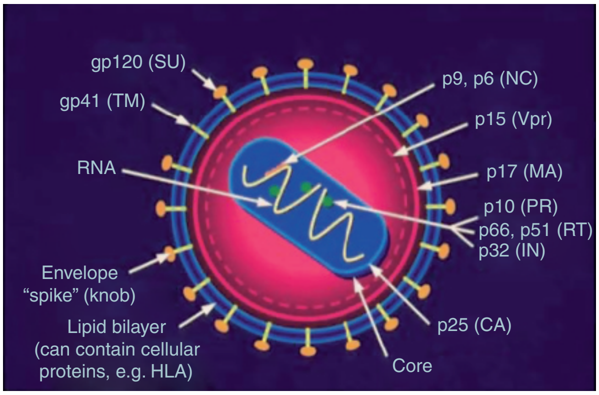

Figure 1 An HIV virion with the structural and other virion proteins identified. The exact locations of Nef and Vif in association with the core have not been well established. The abbreviated viral protein designations are those recommended. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

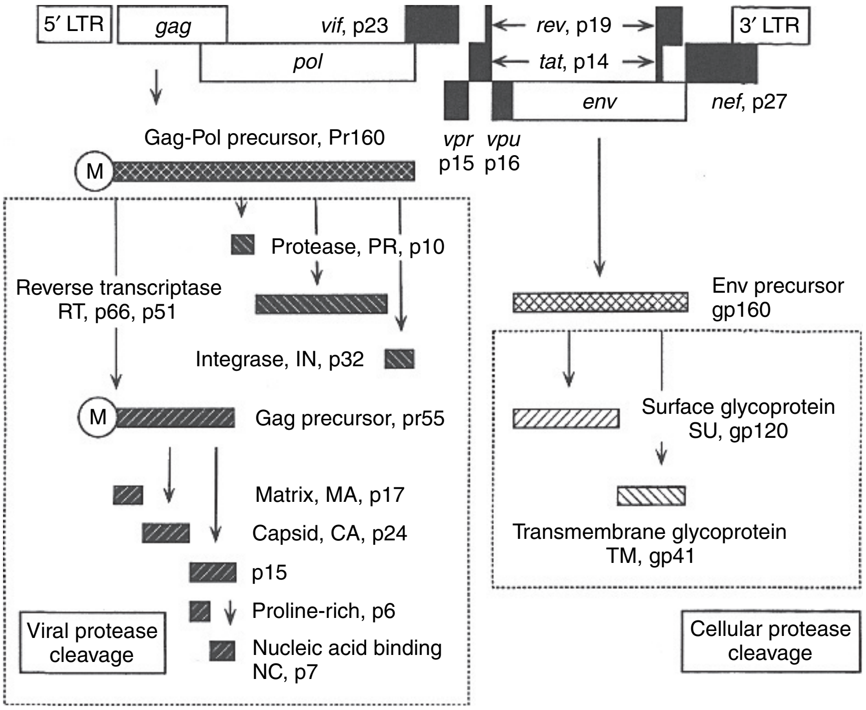
5′ LTR
gag
vif, p23
pol
rev, p19
tat, p14
3′ LTR
env
nef, p27
vpr
p15
vpu
p16
Gag-Pol precursor, Pr160
M
Protease, PR, p10
Reverse transcriptase
RT, p66, p51
Integrase, IN, p32
M
Gag precursor, pr55
Matrix, MA, p17
Capsid, CA, p24
p15
Proline-rich, p6
Nucleic acid binding
NC, p7
Viral protease
cleavage
Env precursor
gp160
Surface glycoprotein
SU, gp120
Transmembrane glycoprotein
TM, gp41
Cellular protease
cleavage

Figure 2 Processing of viral proteins. Some HIV-1 proteins, which are translated from 10 distinct viral transcripts, are further processed by viral and cellular proteases. From 46 translated open reading frames, which include Tev (not diagrammed), 16 viral proteins are made. They form the virion structure, direct viral enzymatic activities, and serve regulatory and accessory functions. The Gag-Pol precursor of 160 kDa is processed by the viral (aspartyl) protease into seven proteins, which include four Gag proteins (MA, p17; CA, p24; late domain, p7; NC, p9), protease (P, p10), reverse transcriptase/RNase (RT, p66, p51), and integrase (IN, p32). The Env precursor (gp160) is processed by a cellular protease into the surface glycoprotein (SU, gp120) and the transmembrane glycoprotein (TM, gp41). Viral regulatory and accessory proteins, which include Tat (p14), Tev (p20), Rev (p19), Nef (p27), Vif (p23), Vpr (p15), and Vpu (p16), are not processed. M, myristoylated. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

cotranslationally modified by the N-terminal attachment of a myristoyl group, which increases its affinity for membranes. It can be observed on Western blot preparations made from whole-cell lysates.

Gag Proteins

The cleavage of the Gag precursor protein (p55) by viral protease (PR) during the process of viral maturation results in three principal proteins: matrix (MA/p17), capsid (CA/p24), and nucleocapsid (NC/p7). The MA/p17 protein constitutes the N-terminal domain of the Gag precursor. The membrane (M)-targeting domain of p55 located within MA is myristoylated on a glycine at its N-terminus by the host cell enzyme N-myristoyl transferase and targets the Gag protein to the membrane. MA is also essential for the incorporation of envelope glycoprotein spikes into mature virions during virus assembly.

The CA (p24) protein sequentially follows MA in the p55 precursor protein. In the mature virion, CA forms the shell of the core, which is occasionally tubular but most often conical, a feature that distinguishes lentiviruses such as HIV-1 from most other retroviruses. It also has crucial roles in particle assembly by binding the cellular cyclophilins in steps following HIV entry into a new target cell. The C-terminal CA domain also includes a stretch of 29 residues, called the major homology region (MHR) because of its conservation among unrelated retroviruses. Genetic analyses have shown that the MHR is essential for retrovirus replication and plays an important role both in viral particle assembly and at postassembly stages.

The NC/p7 protein lies C-terminal to CA in the p55 precursor protein. This hydrophilic protein binds both viral RNA and CA p24 protein, intertwining approximately one molecule with four to six nucleotides of RNA. It increases the proportion of long complementary DNA (cDNA) transcripts produced by reverse transcription. The NC domain harbors two copies of a CCHC-type zinc finger motif, which is essential for specific recognition and packaging of viral genomic RNA into assembling particles. The presence of a p6 domain at the C-terminus of the Gag polyprotein is a characteristic feature of HIV-1 and other primate lentiviruses. Within the Gag precursor, the NC and p6 domains are separated by a peptide called p1. The p6 domain appears to serve primarily as a flexible extension that provides docking sites for cellular factors. Among the Gag domains of different subtypes of HIV-1, the p6 domain is by far the most variable, both in length and in sequence except a P (T/S) APP motif near the N-terminus of p6 and a LXXLF motif near the C-terminus of the domain. The LXXLF motif is essential for the incorporation of the regulatory viral protein Vpr into assembling HIV-1 virions. The late (L) domain of the Gag p55 precursor, located within the NC, involves p6 of the Gag polyprotein that mediates retroviral budding.

The Gag–Pol precursor (p160) is generated by a ribosomal frame-shifting event, triggered by a specific cis-acting RNA motif. The ribosomes shift approximately 5% of the time to the Pol reading frame without interrupting translation, and this frequency of ribosomal frame shifting explains why the Gag and the Gag–Pol precursor proteins are produced at a ratio of approximately 20:1. During viral maturation, the virally encoded protease (PR) cleaves the Pol polypeptide away from Gag and further digests it to separate the protease (p10), RT/RNase H (p66, p51), and integrase (p32) activities. These different enzymes function at different stages of the viral replication cycle and have been the prime targets for antiretroviral approaches. The HIV-1 protease induces the maturation of the viral particle into infectious virions by posttranslational processing of the viral Gag and Gag–Pol polyproteins. The RNA-dependent DNA polymerase (with its RNase H function) acts in the early steps of viral replication to form a double-stranded DNA copy (cDNA) of the viral RNA, which is integrated via the viral integrase into the host chromosomal DNA.

Envelope Proteins

The 160-kDa envelope protein (gp160) is expressed from singly spliced viral mRNA. The envelope protein is first synthesized in the endoplasmic reticulum and glycosylation occurs at asparagine residues during migration through the Golgi complex. The glycosylated gp160 is cleaved in the Golgi complex by cellular proteases into the external surface (SU) envelope protein, gp120, and the transmembrane (TM) protein, gp41. These proteins are transported to the cell surface where noncovalent, labile interactions between the gp41 ecto- and amino domains and discontinuous structures composed of N- and C-termini gp120 sequences in the assembled trimer helps gp120 to adhere to the surface of the virion and infected cells. The long cytoplasmic tail of gp41 appears to be required for HIV envelope glycoprotein incorporation into virions. The virion gp120 located on the virus surface has four regions that are relatively invariant, designated C1 through C4, and has five hypervariable regions, designated V1 through V5, whose amino acid sequences can differ greatly among HIV-1 isolates. The latter characteristic can reflect the evolution of HIV during the course of a single infection and its ability to adapt to drugs and immunologic attack. The third variable region, called the V3 loop, is not directly involved in CD4 binding, but interacts with the HIV chemokine coreceptors CXCR4 and CCR5, thus determining the preferential R5 and X4 tropism of HIV-1. The virion gp120 and gp41 also have major antibody-neutralizing domains (see 'Humoral immune responses to HIV infection').

Regulatory and Accessory Proteins

Splicing events resulting in many subgenomic mRNAs are responsible for the synthesis of other viral regulatory and accessory proteins. One of the regulatory proteins essential for HIV replication is Tat, a transcriptional transactivator encoded by two exons. Tat binds to a short stem–loop structure known as the transactivation response element, which is formed in the 3′ portion of the viral LTR. This attachment stabilizes the nascent mRNA and promotes the elongation phase of HIV-1 transcription, so that full-length transcripts can be produced. The second regulatory protein, Rev (regulator of viral protein expression), is a sequence-specific RNA-binding protein that binds to a 240-base region of a complex RNA secondary structure, called the Rev response element (RRE) located in the viral envelope mRNA. This interaction permits unspliced mRNA to enter the cytoplasm from the nucleus and gives rise to the viral proteins from unspliced and singly spliced mRNAs that are needed for progeny production.

HIV contains four additional genes, *nef, vif, vpr*, and *vpu* (*vpx* in HIV-2), encoding the so-called accessory proteins. Nef (negative factor) protein has been shown to have multiple activities, including downregulation of the cell surface expression of CD4, perturbation of T cell activation, and stimulation of HIV infectivity. Viruses with Nef deleted do not replicate well in PBMCs or *in vivo*. The other accessory gene products, Vpr, Vpu, Vpx and Vif, are involved in virion assembly, cell cycling and budding, and infectivity during the production of infectious viruses. The importance of Vif lies in its countering the intracellular resistance factor APOBEC3G (see 'Intracellular factors').

HIV Genetic Diversity

Globally circulating strains of HIV-1 exhibit an extraordinary degree of genetic diversity, which may influence aspects of their biology such as infectivity, transmissibility, and immunogenicity. This characteristic may be attributed to the infidelity of the viral RT with DNA, suggesting that mutations occur with the DNA template–DNA primer. This enzyme has been found to be highly error prone, resulting in about ten base pair changes in the HIV genome per replicative cycle. One of the early differences to be recognized among various HIV-1 isolates was the variation in sensitivity of the cloned, proviral genome to restriction enzyme digestion. In another approach, DNA heteroduplex analysis on an agarose gel has been used to detect

HIV-1 quasispecies diversity when evaluating different genetic regions (see ‘Detection assays for HIV’).

When the complete genetic sequence data for the initial HIV-1 isolates became available, HTLV-IIIB and LAV were found to be the same isolate whereas SF-2 (formerly called ARV-2) and other HIV-1 isolates were different. Molecular analyses of various HIV isolates reveal sequence variations over many parts of the viral genome, particularly in the envelope region. Currently, on the basis of full-length viral genome sequencing, HIV-1 has been classified into three groups: M (main), O (outlier), and N (Non-M or -O). Eight HIV-2 groups have been identified.

Group M viruses are by far the most widespread and responsible for more than 99% of infections worldwide. Group M viruses have been divided into nine distinct genetic subtypes or clades, designated A to D, F to H, J, and K; and differ among themselves in amino acid composition by at least 20% in the envelope region and 15% in the Gag region. The groups have more than 25% difference in the envelope and Gag regions.

Recombinant viruses are part of the viral genetic diversity. They emerge frequently in human populations where multiple clades co circulate, sometimes becoming an epidemiologically important lineage called as circulating recombinant forms (CRFs). The CRFs are numbered sequentially with the clades involved in recombination or are designated cpx (for complex) if more than four subtypes are involved (http://hiv-web.lanl.gov). The viruses originally identified as subtypes E (the predominant group of viruses involved in heterosexual transmission in Thailand) and I (initially found in Cyprus) are now considered inter-subtype recombinants or CRFs and have been termed CRF-01AE and CRF-04cpx, respectively. In Africa, an inter-subtype recombinant, CRF-02AG, is the dominant virus type.

Clades A through D and the inter-subtype recombinants CRF-01AE and CRF-02AG account for more than 90% of current infections worldwide. Subtype B is dominant in Europe, North and South America, and Australia. Subtypes A, C, and CRF-02AG are responsible for about 75% of new infections occurring globally. Clade C is the most prevalent clade and may represent up to 50% of all HIV infections worldwide. It is found predominantly in South Africa, India, and China (www.unaids.org). Clade D is predominant in Central Africa. CRF-01AE is the most prevalent virus in Southeast Asia. Subtype F includes isolates from Brazil and Romania. Other sequence subtypes (G, H, and I) include viruses from Africa, Russia, and Taiwan. Subtype K, whose *env* C2-V5 sequence branched within group M but remained distinct from all known HIV-1 subtypes, has been reported from Cameroon.

In addition to group M, other isolates initially found in Cameroon and in other African countries at a low frequency are considered outliers and belong to group O. The prototype virus of group N was isolated in Cameroon and appears to be closer to the chimpanzee SIV than either groups M or O of HIV-1.

Eight distinct sequence groups of HIV-2 (A through H) have been identified. Group A in Senegal and Guinea Bissau and group B in the Ivory Coast are the most commonly identified groups. Groups C, D, E, and F came were identified from rural areas in Sierra Leone and Liberia. The HIV-2 viruses appear to be most related to the SIV_{smm} isolates from sooty mangabeys found in the same areas.

HIV Transmission

Overview

After an acute HIV infection, a flu-like illness occurs that can last for up to 3–4 weeks. Sometimes a macular skin rash is also seen (**Table 2**). Those infected people who do not show this acute retroviral syndrome (ARS) usually have a better prognosis. The transmission frequency of a

Table 2 Characteristics of acute HIV infection

Clinical[a]
- Headache, retro-orbital pain
- Muscle aches and joint pains[b]
- Low-grade or high-grade fever[b]
- Swollen lymph nodes[b]
- Nonpruritic macular erythematous rash[b]
- Oral candidiasis
- Ulcerations of the esophagus, anal or vaginal canal
- Acute central nervous system disorders (e.g., encephalitis)
- Pneumonitis
- Diarrhea and other gastrointestinal complaints

Course
- Symptoms usually appear 1 to 4 weeks after acute infection
- Symptoms last from 1–3 weeks
- Lymphadenopathy, lethargy, and malaise can persist for many months
- Generally followed by an asymptomatic period of months to years

Laboratory findings
- First week: lymphopenia and thrombocytopenia
- Second week: lymphocyte number rises secondary to an increase in CD8+ cells; CD4+/CD8+ cell ratio decreases
- Immune activation reflected by increased cytokine levels (e.g., IL-1β, TNFα, and IFNγ)
- Third week: atypical lymphocytes appear in the blood (generally <30%)
- HIV antigenemia and viremia detected within 3–10 days
- Virus can be present in CSF and in seminal fluid within 7–14 days
- Anti-HIV antibodies usually first detected within 1–3 weeks after acute infection
- Proinflammatory cytokines increased in blood (e.g., IL-15 and TNF-α)

[a] Some or all of these findings can be present in the acute retroviral syndrome (ARS). They usually appear after at least 1–4 weeks at peak viremia levels before antibodies are detected.

[b] Most frequently seen.

virus like HIV is greatly influenced by the amount of infectious virus in a body fluid and the extent of contact with that body fluid. Epidemiological studies conducted during 1981–1982 first indicated that the major routes of transmission of AIDS were intimate sexual contact and contaminated blood. The syndrome was initially described in homosexual and bisexual men and intravenous drug users but its transmission through heterosexual activity was soon recognized. High-risk sexual behavior early in the epidemic caused the rapid increase in HIV transmission. Subsequent studies showed that transfusion recipients and hemophiliacs could contract the virus from blood or blood products, and infected mothers could transfer the causative agent to newborn infants. Surveillance and epidemiologic data throughout the world continue to support strongly three primary modes of transmission: sexual contact (heterosexual and homosexual); exposure to blood, largely through injecting drug use and transfusion; and perinatal transmission from infected mothers to their infants.

These means of transmission can be greatly explained by the relative concentration of HIV in various body fluids. Blood has high levels of both infectious virus and virus-infected cells; however, the amount of infectious virus is less than the number of HIV-infected cells that could transfer virus to an individual. HIV-infected cells in genital fluids appear to be a major source of transmission by the sexual route. Sexually transmitted diseases further enhance the risk of virus transmission to either partner by increasing the presence of virus-infected cells. Saliva is not a major source of transmission as it contains HIV-inhibitory substances and only small amounts of infectious virus. Transmission of virus from mother to child can involve direct infection of the fetus *in utero* or exposure of the newborn to maternal blood and secretions during birth. The major factors affecting this transmission are the levels of infectious virus in the mother at the time of delivery; amniotic fluid; and uterine, placental, and fetal tissues. The number of virus-infected macrophage and T cells in mother's colostrum and milk also determines this transmission.

Effective preventive measures for sexual transmission include condoms. Female condoms, diaphragms, vaginal microbicides, or compounds that inhibit HIV adsorption and cell-to-cell contact need further evaluation. Most recently, male circumcision has been shown to decrease transmission in men by up to 60%. Treatment of ulcerative genital diseases that can enhance HIV transmission also reduces infection. Highly active antiretroviral therapy (HAART) is being evaluated for use in postexposure prophylaxis (PEP). Antiretroviral therapy can also greatly reduce the risk of mother–child transmission.

Cells Are Involved in HIV Transmission

An important consideration in understanding HIV transmission is the role of the virus-infected cell in transmitting HIV not only to immune cells but also to macrophage and mucosal-lining cells. These infected cells (lymphocytes and macrophage) are found in genital fluids. Several electron microscopy studies have shown that, whereas HIV alone may not directly infect cultured cervical-lining cells or mucosal cells from the bowel, infected T cells and macrophage can efficiently deliver virus to these cells. Time-lapse photography has shown that the infected cells, remaining viable, can move from one mucosal-lining cell to the other, delivering virus. They thus emphasize this important means of transmission. Infection of the bowel mucosa through HIV-infected cells in genital fluid could account for the infected cells seen in the mucosal-lining cells of the bowel.

Other work in this field during the late 1980s and early 1990s indicated the wide cellular host range of HIV, infecting several cell types of the brain, as well as the bowel, heart, kidney, liver, testes, prostate, and most likely many other tissues (**Table 3**). Virus replication in lymphoid tissues mirrors the progression of HIV infection in individuals – destruction of the germinal centers in the lymph node, accompanied by increased virus expression in lymphoid tissues. In some cases, HIV-1 can also infect brain and alveolar macrophage in a CD4+-independent manner.

Detection Assays for HIV

Various assays for the detection of HIV have been developed since the recognition of AIDS. The virus' presence in cell culture can be determined by RT activity. Immunofluorescence assays, ELISAs, and the Western blot help to detect antibodies to HIV. All these tests are confirmatory assays for HIV infection. Other procedures like measurement of viral p24 antigen (directly and after acid dissociation) and of HIV RNA levels by reverse transcription-PCR have increased the sensitivity for detection of HIV in blood and other body fluids. Even though these procedures do not distinguish between infectious and noninfectious virions, they have been particularly useful in monitoring the effect of antiviral treatment in HIV-infected individuals.

The development of flow cytometry in the 1970s greatly helped to study the effect of HIV on the immune system as it enabled clinical and research laboratories, via selective monoclonal antibodies, to determine the number of CD4+ and CD8+ cells in humans. While the CD4+/CD8+ cell ratio is usually 2:1, it was very

Table 3 Human cells susceptible to HIV infection[a]

- Hematopoietic
- _B cells
  - Bone marrow endothelial cells
  - Dendritic cells (DCs)
  - Eosinophils
  - Follicular DCs
  - $\gamma\delta$ T cells
  - Macrophage
  - Mast cells
  - NK cells
  - T cells
  - Megakaryocytes
  - Promyelocytes
  - Stem cells
  - T lymphocytes
  - Thymic epithelium
  - Thymocytes
- Brain
  - Capillary endothelial cells
  - Astrocytes
  - Macrophage (microglia)
  - Oligodendrocytes
  - Choroid plexus
  - Ganglia cells
  - Neuroblastoma cells
  - Glioma cell lines
  - Neurons (?)
- Skin
  - Fibroblasts
  - Langerhans cells (LCs)
- Bowel
  - Columnar and goblet cells
  - Enterochromaffin cells
  - Colon carcinoma cells
- Others
  - Myocardium
  - Renal tubular cells
  - Synovial membrane
  - Hepatic sinusoid endothelium
  - Hepatic carcinoma cells
  - Kupffer cells
  - Dental pulp fibroblasts
  - Pulmonary fibroblasts
  - Fetal adrenal cells
  - Adrenal carcinoma cells
  - Retina
  - Cervix-derived epithelial cells
  - Prostate
  - Testes
  - Urethra
  - Osteosarcoma cells
  - Rhabdomyosarcoma cells
  - Fetal chorionic villi
  - Trophoblast cells

[a] Susceptibility to HIV determined by *in vitro* and *in vivo* studies.

soon recognized that the ratio in infected individuals often was reduced to less than 1. The number of CD4+ T cells, usually in the range of 600–1200 cells μl^{-1}, became reduced over time, particularly in progressors, to the low hundreds (e.g., <300 cells μl^{-1}). These findings supported the observation that the CD4+ lymphocyte was a major target for HIV replication and cell death.

HIV Infection and Replication

Virus–Receptor Interactions

Primary receptor: CD4 molecule

One of the first breakthroughs in the studies of HIV came with the discovery that its major cellular receptor was the CD4 molecule, thus, explaining its preferential growth in CD4+ lymphocytes. Subsequent crystal studies of CD4 revealed that the binding site for the viral envelope glycoprotein 120 (gp120) was located on a protuberant ridge along one face of the D1 region of the CD4 molecule (in the complementarity-determining region 2 (CDR2) domain). This binding site appears to overlap with the major histocompatibility complex (MHC) class II-binding site, thus, affecting the use of inhibitors of CD4–gp120 interaction. The fourth conserved portion (C4) near the carboxyl terminal end of the gp120 acts as the major viral region binding to the CD4. The CD4-binding domain of gp120 also includes hydrophobic and hydrophilic domains in conserved C2, C3, and C4 regions that are involved in the conformational structure of the envelope. Glycosylation of the envelope gp120 is another potential factor influencing the CD4–gp120 interaction. After attachment to the CD4 molecule, gp120 appears to be displaced, either completely or partially, or cleaved by cellular proteases as observed with X4 viruses. This process leads to conformational changes in the envelope and uncovering of domains in gp41 that are needed for virus–cell fusion.

Certain CD4+ T cell lines and undifferentiated CD4+ monocytes were not found susceptible to HIV and some CD4– cells could be infected. Therefore, the CD4 receptor alone did not appear to be sufficient or the sole mean for viral attachment and subsequent entry. Hence, the existence of other cell surface receptors for HIV was proposed.

Secondary receptors for HIV infection

Several chemokine receptors, belonging to a seven-membrane-spanning protein family, most importantly CXCR4 and CCR5, have been found to act as secondary receptors for HIV entry. The affinity of these second coreceptors to HIV isolates varies and can account for at least two distinct biologic phenotypes of HIV. The X4 or T cell-line-tropic viruses recognize CXCR4 as the coreceptor, and R5 or macrophage-tropic strains bind to the CCR5 coreceptor. Some dual tropic HIV isolates (R5/X4) that are both macrophage-tropic and T cell-line-tropic use either coreceptor.

CXCR4

CXCR4 acts as a coreceptor for T cell-line-tropic HIV strains, permitting a closer interaction between the virus and the cell surface. The amino terminal domain of CXCR4 is involved in HIV binding, especially the second extracellular loop structure. The viral V3 loop is involved in X4 virus infection. The natural ligand for CXCR4 is the chemoattractant stromal-derived factor 1 (SDF1), which can block HIV infection of T cells. The X4 strains induce multinucleated syncytia in T cell lines like MT-2, and hence are called 'syncytia-inducing (SI) viruses'.

CCR5

CCR5 acts as a coreceptor for macrophage-tropic HIV isolates. The β-chemokines RANTES, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β efficiently block infection by macrophage-tropic HIV strains at the CCR5 receptor site, presumably by competitive interaction. The amino terminal or first extracellular region of CCR5 takes part in the interaction with a highly conserved portion of gp120, which is located between the V1, V2, and V3 loops. The R5 strains do not usually induce multinucleated syncytia in T cell lines, and thus are considered 'nonsyncytia-inducing (NSI) viruses'. A 12-bp deletion in CCR5 that introduces a frameshift mutation results in a protein lacking the C-terminal domain (CCR5 Δ32). Cells with a genetic variant homozygous for this mutant chemokine receptor allele are resistant to HIV infection with R5 viruses.

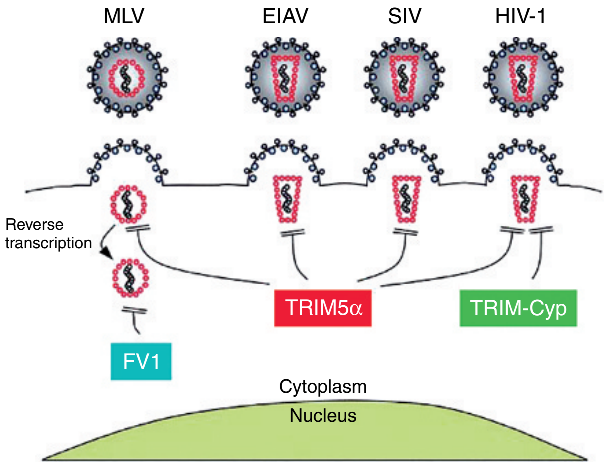

Figure 3 Capsid-specific restriction factors. After entry into the cytoplasm, retroviral capsids can be recognized and infection blocked by one of many factors. Fv1 is unique to the mouse and blocks infection by MLV only, in an Fv1 allele- and MLV strain-specific way. TRIM5α, which is present in most primates, can block infection by a range of retroviruses including N-tropic MLV, equine infectious anemia virus (EIAV), simian immunodeficiency virus (SIV), and HIV-1. The precise spectrum of TRIM5α antiretroviral activity depend on its species of origin. A unique form of TRIM5 exists in owl monkeys, due to transposition of a cyclophilin A (CypA) pseudogene, and the resulting fusion proteins inhibits HIV-1 because of the latter's CypA-core binding activity. Reproduced from Bieniasz (2004) *Nature Immunology* 5: 1109–1115 with permission from Nature Publishing Group.

Virus–Cell Fusion and Entry

The conformational changes induced in the envelope after attachment to the CD4 molecule and coreceptors combined with the displacement or cleavage of gp120 exposes the domains on the envelope gp41 that are needed for virus–cell fusion. Another conformational change in gp41 causes insertion of the N-terminal hydrophobic fusion peptide region into the phospholipid membranes of the target cell. This process could lead to a conformational change in CD4 as well as dissociation of the envelope gp120 from the virion surface. Subsequently, the viral nucleocapsid enters into the target cell cytoplasm in a pH-independent manner. This process can involve different viral and cellular proteins.

Virus Replication, Assembly, and Release

After HIV has entered the host cell as a ribonucleocapsid, several intracellular events lead to the integration of a proviral form into the cell chromosome (**Figure 3**). The HIV RNA exits from the viral capsid into the cytoplasm of the host cell. It undergoes reverse transcription by using its RNA-dependent DNA polymerase and RNase H activities to form a double-stranded DNA copy (cDNA) of its genome. The NC/p7 Gag protein helps in efficient reverse transcription by increasing the proportion of long-cDNA transcripts and later plays a role in the efficient packaging of genomic RNA.

The synthesized double-stranded HIV genome is actively transported to the nucleus in a preintegration complex (PIC), which can include the viral proteins PR, RT, IN, and Vpr. The noncovalently bound circular forms of viral DNA randomly integrate into the transcriptionally active regions of the host cell chromosomal DNA, particularly those activated by HIV infection. Proviral DNA is replicated as part of host cell genome and may persist in this form through many rounds of mitotic cell division. The earliest mRNAs made in the cell are doubly spliced transcripts encoded by major regulatory genes, particularly *tat, rev*, and *nef.* High-level expression of Tat and Nef proteins increases viral replication. The Rev protein encourages the transport of large, unspliced mRNA, which is responsible for viral structural gene products and enzymes, into the cytoplasm.

Assembly of mature viral particles occurs at the cell membrane where viral RNA is incorporated into capsids that bud from the cell surface, taking up the viral envelope protein. Two mechanisms of budding have been proposed. In the first model, the Gag and Gag–Pol precursors localize to 'rafts' on the plasma membrane that are enriched in sphingolipids and cholesterol. The Gag precursor protein, p55, associates with the cytoplasmic domain of the envelope transmembrane protein gp41, which in turn binds to the viral gp120 on the outer surface. As they bud through the host cell membrane, virus particles acquire a lipid bilayer that contains envelope molecules distributed as trimers and oligomers on the membrane. The other Trojan exosome hypothesis, considered common to macrophage, proposes that HIV assembly takes place in multivesicular bodies that come to the cell surface and fuse with the plasma membrane, releasing virus as an exosome. The virus recruits cellular proteins to break the cell membrane. During or shortly after budding, the viral protease (PR) cleaves Gag and Gag–Pol precursor proteins to their mature products, generating infectious virions. The released viral particles complete the replication cycle by subsequent infection of a new host cell.

Differences in Virus Production

Intracellular factors

Various intracellular factors can influence the extent of productive viral infection as noted by differences in virus production in PBMCs. These factors may also be responsible for the lack of replication of some X4 viruses in macrophage and R5 viruses in T cell lines. Intracellular blocks can affect early and late steps of reverse transcription, formation of PIC, and transport of the PIC to the cell nucleus (see titled 'Virus replication, assembly, and release'). The early mRNA transcription appears to be dependent primarily on the binding to the HIV LTR by cellular transcription factors like nuclear factor kappa B (NF-κB), NFAT, AP-1, Sp-1, and Tat-binding proteins. Activation is important for HIV replication in T cells as it involves the conventional interaction of intracellular factors with regions in the viral LTR. Differentiated macrophage compared to monocytes are most susceptible to virus replication due to upregulation of NF-κB transcriptionally active proteins.

Certain cytokines like TNF-α have been shown to affect intracellular transactivating factors within the LTR, particularly NF-κB, and can substantially increase HIV production. The binding of HIV proteins like Tat to the TAR element of the viral LTR in conjunction with cellular RNA-binding proteins subsequently upregulates viral expression as the cellular proteins increase Tat binding to the TAR region.

Natural cellular resistance to HIV

Major observations made over the last few years have shown the presence of natural mechanisms within cells that might influence the extent of virus production in resting CD4+ cells. These cellular resistances were recognized because they were sometimes countered by HIV proteins. APOBEC3G, a species-specific, cytosine deaminase, induces G-to-A mutations in newly synthesized viral DNA leading to the replacement of cytosine by uracil in retroviral minus strand DNA. This change creates an increased frequency of G-to-A mutations in the plus strand DNA and results in inactivated progeny viruses either because of mutations or because of DNA degradation triggered by viral N-glycosidases. Recent studies also suggest that APOBEC3G and -3F can interact with the HIV-1 integrase and inhibit proviral DNA formation. APOBEC3G and -3F are countered by the viral Vif protein, which prevents these intracellular proteins from functioning in the infected cell and ensures production of infectious virions. Most recently, virus replication block in resting CD4+ cells has been linked to the specific location of APOBEC3G in the cell.

As another example, the restriction of HIV in monkey cells has been shown to be related to the presence of an intracellular protein, TRIM5α, which appears to block the opening of the HIV capsid within the cell and affects reverse transcription and the formation of the PIC. TRIM5α may also restrict HIV progeny production by degrading the Gag protein before core formation.

In other studies, a gene product, Murr-1, has been shown to inhibit the degradation of Iκ-Bα, and therefore can play a role in creating and maintaining latency (silent HIV infection) within the cell. Moreover, an yet to be identified human cellular restriction factor inhibits HIV assembly and appears to be counteracted by Vpu. All these factors could offer novel approaches to antiretroviral therapy.

Virus Infection of Quiescent Cells and Viral Latency

In a classic state of viral latency, the full viral genome is in the cell but expression is completely suppressed. However, as seen in other retroviruses, HIV can infect and remain in an unintegrated state for several days without evidence of virus replication. Viruses may then replicate to very low levels or remain latent. The various mechanisms possible for this cellular latency, or 'silent virus' state, have not been fully elucidated but could be related to methylation of viral DNA or direct activity of host cellular products like APOBEC3G and Murr-1 on the HIV genome. The induction or activation of HIV from a latent state can be achieved by a variety of approaches including irradiation, halogenated pyrimidines, and coinfection of a cell with other viruses.

The level of cell activation as measured by the expression of HLA-DR and CD25 can greatly influence the extent of viral reverse transcription and release of infectious virus from the cell. Early *in vitro* studies using nondividing peripheral blood CD4+ T cells have shown that the nonactivated (no HLA-DR and low CD25 expression) quiescent or resting CD4+ T cells cannot be productively infected by HIV. The virus can enter CD45RA+ naive CD4+ T cells, but limited transcription of the viral genes takes place and no viral proteins are formed as the virus is not integrated. Upon activation, full-length viral DNA is formed and integrated, followed by virus production. However, without activation, the infection is aborted.

These observations with purified quiescent CD4+ cells have been applied to clinical specimens. Purified CD4+ cells from asymptomatic individuals consist of a large population of resting cells lacking HLA-DR expression that contain viral DNA. In subjects on HAART, a reduced number of latent CD4+ T cells have been reported; a small percentage of these cells will replicate the virus when treatment is stopped. These latent cells are established early in acute infection even in treated subjects. A recent study estimates the half-life of the latent viral reservoir to be 4.6 months in individuals who initiate antiviral therapy early in HIV infection. The study projects that it may take approximately 8 years of continuous antiviral therapy to completely eliminate this reservoir of latently infected resting CD4+ T cells in such individuals. Thus, cycles with fluctuations in virus infection and loss characterize a dynamic situation within an infected individual (**Figure 4**).

Clinical latency defines the absence of symptoms of HIV infection although low-level virus replication can remain active in the host. Long-term survivors (LTS) are identified as individuals who have been HIV infected for more than 10 years but do not show any symptoms and do not develop disease (see 'Factors affecting HIV disease progression'). Apart from the cellular factors, the immunologic response of the host against the virus influences this clinical condition. Thus, biologic and clinical latency can be quite distinct.

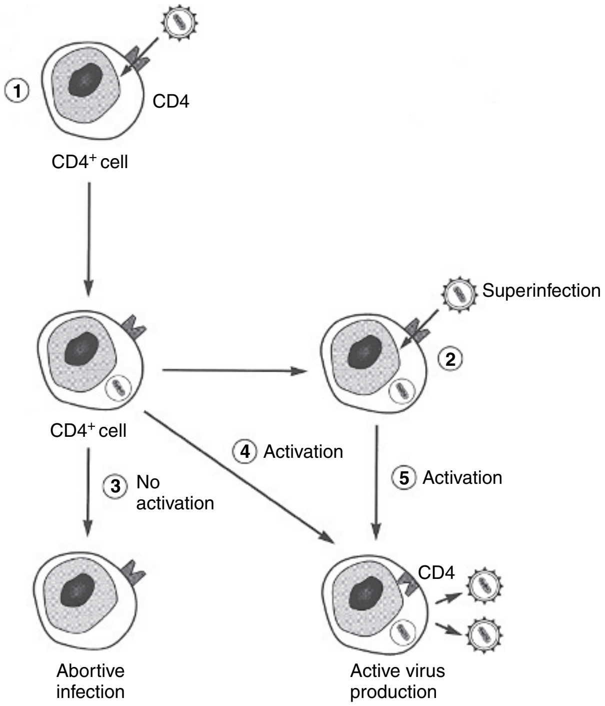

Figure 4 HIV can infect a resting CD4+ cell that expresses the CD4 molecule (step 1). If that cell is not activated, virus replication cannot take place. Because the CD4 molecule is still expressed on a resting cell, superinfection by another virus can occur (step 2). With no activation of the resting cell after infection, an abortive infection takes place (step 3). With activation, active virus replication takes place (step 4). Similarly, activation of the superinfected cell can lead to virus production and both type viruses or recombinants might be found. Following activation and virus production, the CD4 molecule is down-modulated so superinfection cannot occur. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

Effect of HIV on Cells and the Immune System

Overview: HIV Cytopathology

An important part of understanding of HIV pathogenesis comes from studying the cytopathic effects of the virus or its proteins on individual host cells. Several processes are involved in induction of cell death by HIV, including syncytium formation, cell membrane changes, necrosis, and apoptosis. Indirect viral effects involve syncytia formation as a result of fusion of infected cells with uninfected CD4+ cells; it can be the first sign of HIV infection of cultured PBMCs. HIV, particularly X4 biotypes, induces cell–cell fusion by the interaction of its envelope gp120 and gp41 proteins with CD4+ cells. This virus envelope–cell membrane fusion causes cell death by changes in cell membrane integrity that permit an influx of monovalent and divalent cations with water, leading to balloon degeneration. The cell–cell fusion is temperature-dependent and involves surface carbohydrates and glycolipids as well as certain surface adhesion molecules like LFA-1 and CD7 glycoprotein. Accumulation of unintegrated viral DNA in the cell cytoplasm can also be responsible for cell death.

Apoptosis or programmed cell death requires cell activation, protein synthesis, and the function of a Ca^{2+}-dependent endogenous endonuclease that fragments the cellular DNA into small nucleotide units. Apoptosis of host cells results from the direct action of viral proteins (e.g., Nef, Vpu, Vpr, and Tat), gp120 binding to the CD4 molecule, disorders in antigen-presenting cells (APCs), and superantigens. HIV gp120 and Vpr can cause apoptosis by blocking replication at the G_2 stage of cell cycle. HIV-induced apoptosis of CD4+ cells may occur either due to direct infection of CD4+ cells or due to indirect effects of HIV, which include interference with T cell renewal by HIV, bystander killing induced by HIV gene products, and activation-induced cell death and activated T cell autonomous death. HIV-induced activation makes CD4+ and CD8+ cells more susceptible to apoptosis. These cells are characterized by increased expression of cell surface markers like HLA-DR, CD38, and CD69, mainly on memory (CD45RO+) CD4+ and CD8+ cells. Superantigens can also induce cell activation associated with apoptosis. Apoptosis is more common in uninfected (bystander effect) rather than infected CD4+ cells. The process can be observed in other cells, including CD8+ T lymphocytes, B lymphocytes, and neuronal cells.

HIV infection and viral proteins can disrupt production of cytokines by the immune cells, thus interfering with their normal immune function. For example, the gp41 envelope protein induces IL-10 secretion by monocytes and macrophage and can decrease IL-2 and interferon-γ (IFN-γ) production by CD4+ and CD8+ T cells, which are important for cell-mediated immunity. The cytokine alterations also influence apoptosis as type 1 cytokines prevent the process and type 2 cytokines increase apoptosis. Cytokines can cause cell death via enhanced HIV replication or by direct toxicity.

CD4+ T Cell Depletion

A marked reduction in CD4+ cell number and function is the primary HIV-induced immune abnormality. Various mechanisms are responsible for the CD4+ cell loss, including direct cell destruction by virus or its proteins, immune activation with apoptosis, loss of *de novo* cell production, and reduced cell proliferation (**Table 4**).

Effect of HIV on CD4+ T cell number

In HIV-infected individuals, the number (2×10^{11}) of mature CD4+ T cells as observed in healthy adults is halved by the time the peripheral blood CD4+ T cell count falls to 200 cells μl^{-1}. The loss of CD4+ lymphocytes of the helper (inducer) cell type can also be reflected in the observed inversion of the CD4+ T cell/CD8+ T cell ratio in HIV infection. One reason for the reduction in these circulating CD4+ T cells is that these cells remain in the tissue after activation within the lymph node as compared to circulating CD8+ T cells. Moreover, HIV can elicit expression of CD62L and CCR7 on CD4+ T cells, thus trafficking them back to lymph nodes. Additionally, direct HIV infection of CD4+ T cells decreases their numbers. The disruptions in CD4+- and CD8+ T cell repertoire also reflect CD4+ T cell number reduction.

Importantly, the level of CD4+ T cells in the blood may not reflect the true number in infected individuals, since the most extensive CD4+ T cell loss is in the gastrointestinal tract. This process results from early infection with R5 viruses and later by ongoing infection of the CD4+ T cells, apoptosis, and cytotoxic T cell responses. In contrast, naive and memory CD4+ T cells predominantly found in lymph nodes are targeted primarily by X4 viruses.

Another explanation for the low levels of CD4+ T cells is that the enhanced CD4+ T cell destruction is not compensated by an increased production of these cells, either by replication of effector memory cells present in tissues or by production of naive cells derived from thymus. Efficient thymopoiesis appears to be important even after therapy for natural maintenance and recovery of naive and total CD4+ T cells. This decrease in production of CD4+ T cells is further supported by cell-labeling studies, which have demonstrated that the absolute production rate (10 CD4+ T cells μl^{-1} per day) of CD4+ T cells does not increase during HIV infection. Advanced HIV infection is also characterized by a shortened average life span of T cells. In HIV-infected subjects with CD4+ T cell levels of ≥ 350 cells μl^{-1}, the CD4+ T cell half-life is about 30% less as compared to the half-life of about 84 days found in healthy individuals. The total T cell half-life has been shown to increase with HAART in association with enhanced cell production as reflected by abundant thymic tissue.

Table 4 Potential factors involved in HIV-induced loss of CD4+ lymphocyte number and immune function[a]

- Direct cytopathic effects of HIV and its proteins on CD4+ cells and progenitor cells
- Induction of apoptosis via immune activation
- Cytokine cytotoxicity
- Effect of HIV on production of cytokines needed for CD4+ cell function
- Destruction of bone marrow (e.g., stem cells and stromal cells) and lymphoid tissue (e.g., thymus); lack of lymphopoiesis
- Cell destruction via circulating envelope gp120 attachment to normal CD4+ cells: ADCC and CTL
- CD8+ cell cytotoxic activity against uninfected CD4+ cells

[a] ADCC, antibody-dependent cellular cytotoxicity; CTL, cytotoxic T lymphocytes.

Effect of HIV on CD4+ T cell proliferation

HIV-induced immune activation also causes rapid proliferation of CD4+ and CD8+ T cells, resulting in cell death, most likely by activation-induced apoptosis. In addition, a reduced ability of CD4+ cells to proliferate in response to HIV proteins and decreased CD4+ cell proliferative responses to T cell receptor (TCR) antibodies have been observed in HIV-infected individuals, particularly with high viral load. HIV infection is also associated with decreased expression of IL-7 receptor α (IL-7R α), and thus, decreased sensitivity to IL-7-induced cell proliferation.

Effect of HIV on CD4+ T cell function

The CD4+ T cell function, measured by the proliferative responses to specific stimuli, is also compromised in HIV infection. These cells from HIV-infected subjects have a reduced response to recall antigens such as flu, followed by a decreased proliferative response to alloantigen (MHC), and finally a loss of response to lectins like phytohemagglutinin. The latter subjects progress to disease faster than those who have all or some of these immunologic functions. Early initiation of antiretroviral therapy in acute infections may help to restore these immunologic functions. HIV infection of CD45RO+ memory CD4+ T cells makes them more susceptible to the cytotoxic effects of HIV, thus causing immune dysfunction.

Indirect effects of HIV on CD4+ T cell number and function

HIV infection of CD4+ T cells can lead to disorders in cytokine production, thus affecting the T helper (T_H) function of CD4+ T cells (see 'T lymphocyte immune responses to HIV infection'). HIV can also remain in a latent state in a large number of CD4+ cells, particularly in asymptomatic individuals, thus affecting the function, long-term viability, and growth of these cells (see 'Virus infection of quiescent cells and viral latency'). It may reduce cell proliferation and increase their sensitivity to toxic effects of cytokines.

Table 5 Components of the innate and adaptive immune systems

Innate immune system
Dendritic cells (DCs)
Macrophage
Neutrophils
NK cells
$\gamma\delta$ T cells
NK T cells
Plasmacytoid dendritic cells (PDCs)
B-1 cells
Cytokines (e.g., interferon)
Chemokines
Antimicrobial peptides: defensins, cathelicidins, pentraxins
Complement
Lectin-binding proteins (collectins)
CD8+ T cells[a]
Adaptive immune system
Dendritic cells (DCs)
Macrophage
B lymphocytes
CD4+ T lymphocytes
CD8+ T lymphocytes[b]

[a] Noncytotoxic antiviral activity.
[b] Cytotoxic activity.

Host Immune Responses to HIV

Innate Immune Responses in HIV Infection

The innate immune system represents the first line of defense against infectious organisms (see 'Innate immunity'). It is very important for preventing and maintaining control of HIV infection. This immunity differs in several ways from adaptive immunity; they have different cellular and soluble participants (**Table 5**). Innate immune cells respond rapidly to pathogens and can act without MHC restriction. They recognize specific conformational patterns of organisms rather than a specific epitope. Importantly, the cytokines (e.g., IFN-α) produced by innate immune cells can have direct antipathogen effects. Some of the components of the innate immune system are discussed in the next section.

The innate immune system recognizes incoming microbial organisms via evolutionary conserved pathogen-associated molecular patterns (PAMPs) and responds through intracellular signaling with cytokine production. The pathogen recognition receptors (PRRs) serve as pathogen sensors. PRR signaling activates transcription factors like NF-κB and IFN regulatory factors 3 and 7 (IRF3 and IRF7), which induce inflammatory responses and stimulate the immune system or elicit the production of type 1 IFNs (e.g., IFN-α). These PRRs include the following:

1. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) participate mainly in the recognition of bacterial pathogens and their products (e.g., muramyl dipeptide peptidoglycans). They are located intracellularly and after ligand interaction, activate NF-κB, leading to the expression of cytokines and chemokines.
2. Retinoic acid-inducible gene 1 (RIG-1)-like-receptors (RLRs) are RNA helicases that recognize double-stranded RNA of viruses, which enter by endocytosis or fusion, and elicit cytokine production for elimination of replicating viruses.
3. C-type lectin-like receptors (CLRs) include β-glucan and mannose receptors, which interact with mannose-containing organisms and encourage engulfment by macrophage.
4. Toll-like receptors (TLRs) are the most notable class of PRRs. They are type 1 integral membrane glycoproteins with cytoplasmic, transmembrane, and extracellular domains. There are currently 11 identified human TLRs that recognize PAMPs extracellularly (e.g., TLR-1, -2, -4, and -6) or intracellularly (e.g., TLR-3, -7, and -9). They recruit signal transduction molecules like MyD88, leading to activation of transcription factors like NF-κB and expression of cytokines (e.g., IFN-α). TLRs can be found on macrophage, dendritic cells (DCs), neutrophils, B cells, epithelial cells, endothelial cells, and T cells. A recent study indicates that astrocytes in the brain can also participate in local innate immune responses via TLRs.

Dendritic cells

DCs play an important role in innate immunity by their induction of immune responses. A variety of subtypes of these cells are distributed throughout the body (**Table 6**). DCs take up antigens efficiently and present them to the immune system via MHC–peptide complexes on their cell surface. They are the only cells capable of initiating a primary immune response by stimulating naive T cells to proliferate. They link innate and acquired immunity by stimulation of naive T lymphocytes and natural killer (NK) cells and by release of various cytokines (e.g., IL-12, IL-10, and IFN-α).

Two major types of blood DCs have been recognized: myeloid dendritic cells (MDCs) and plasmacytoid dendritic cells (PDCs) (**Table 7**). MDCs are primary producers of IL-12. They mature into APCs after exposure to IFN-γ (secreted by NK cells or CD8+ cells) or IFN-α (secreted by PDCs). Subsequently, they migrate into various tissues and play a role in inducing both innate and adaptive immune responses.

Table 6 Distribution of dendritic cells (DCs)

DC type	*Location*
Langerhans cells (LCs)	Skin and genital tract
Interdigitary cells	Lymph node
Follicular DCs	Lymph nodes
DCs	Thymus
Interstitial DCs	Heart, lung, and intestine
MDCs and PDCs	Blood

All these DCs are susceptible to HIV infection to a varying extent (see ‘Cells are involved in HIV transmission’ and ‘Dendritic cells’). MDC, myeloid DC; PDC, plasmacytoid DC.

Table 7 Comparison of blood dendritic cells (DCs)

Marker	*PDC*	*MDC*
Lineage	–	–
CD11c	–	+
CD4	++	+
HLA-DR	++	++
CCR5/CXCR4	+	+
CCR7	±	–
IL-3R (CD123)	++	–
BDCA-2, -4	++	–[a]
DC-SIGN	–	–[b]
Growth factor	IL-3	GM-CSF
Phagocytosis	+/−	+
TLR-7, -9	+	–[c]
TLR-2–6, -8	–	+
Major cytokine production	IFN-α TNF-α β Chemokines	IL-12

Only monocytes

GM-CSF, granulocyte–macrophage colony-stimulating factor; MDC, myeloid dendritic cells; PDC, plasmacytoid dendritic cells; TLR, toll-like receptor.

[a] Some expression of blood dendritic cell antigen 4 (BDCA-4) can be detected.

[b] Expressed on monocyte-derived dendritic cells (MDDCs).

[c] MDDCs are similar to MDC but larger in shape and have a dominance of C type lectin receptors and some evidence of TLR-7 expression.

PDCs were initially recognized as CD4+ CD11c− Lin− cells that grow in the presence of Flt3 ligand. Subsequently, they were found to be the principal producers of IFN-α. The addition of CD40L to PDCs results in their differentiation into mature DC. PDCs can be identified by the expression of blood dendritic cell antigen 2 (BDCA-2) and BDCA-4, now classified as CD303 and CD304, respectively. Antibodies to these specific markers are routinely used to purify and identify PDC population.

PDCs are found in the T cell associated region of lymphoid tissue and have plasma-cell morphology, representing 0.2–0.8% of PBMCs. PDCs develop from hematopoietic stem cells in the fetal liver, thymus, and adult bone marrow, circulate in blood, and migrate to lymphoid tissues and inflammatory sites as they express chemokine receptors (e.g., CXCR4, CD62L, and CCR7).

PDCs recognize pathogens via TLR-7 and TLR-9 located in endosomes. The ligands for TLR-7 and TLR-9 are single-stranded RNA viruses and unmethylated CpG rich oligonucleotides, respectively. Upon pathogen recognition, PDCs produce large amounts of type 1 IFNs (1–2 U or 3–10 pg per cell), which is 200–1000 times more than that produced by any other blood cell type. Type 1 IFNs play an important role in HIV infection. They can directly interfere with virus replication, increase the activity of NK cells, and increase the function of MDCs as APCs by upregulating MHC and costimulatory molecules on DCs. Increased MHC class I expression also enhances cytotoxic T cell activity. Additionally, type 1 IFNs increase IFN-γ production by CD4+ T cells and promote a type 1 cellular immune response. Upon maturation into DCs with CD40 ligand interaction with CD4+ T cells, PDCs can become DC-2 cells that produce various cytokines like IL-4, IL-5, and IL-10, and enhance antibody production (a type 2 immune response).

Dendritic cells and HIV infection

The DCs play a major role in initial HIV infection and dissemination as they are potentially the first cells to be infected by HIV in the genital mucosa. A variety of C-type lectins on DCs, importantly DC-SIGN, are involved in uptake of HIV by endocytosis, and subsequently direct infection of CD4+ cells takes place as HIV is passed to them by DCs in the lymphoid tissue. It has been assumed that Langerhans cells (LCs) in the genital mucosa mediate the transmission of HIV-1 to CD4+ T cells through the C-type lectin langerin. However, a recent study shows that langerin can internalize HIV-1 and prevent transmission. This possibility needs further study. The DCs from lymph nodes, blood, and myocardium are also susceptible to HIV infection (**Figure 5**).

Apparently, selective subpopulations of blood DCs are susceptible to HIV infection. The monocyte-derived dendritic cells (MDDCs) obtained after cytokine (e.g., GM-CSF) treatment *in vitro* and MDCs are susceptible to HIV infection. Immature MDCs are more susceptible as they express CD4 and high levels of CCR5. Moreover, they can also engulf virus without the need of CD4+ cell interaction. Low-level replication of HIV in mature and immature MDCs acts as a reservoir for transfer of virus to activated CD4+ cells. MDCs replicate R5 virus isolates better than X4 viruses. PDCs are susceptible to both X4 and R5 virus infection via CD4 and other coreceptors as they express both CXCR4 and CCR5. However, the extent of HIV replication resembles that observed in unactivated CD4+ cells. However, on maturation with CD40L, the virus replicates to higher levels with noted cytopathic effects.

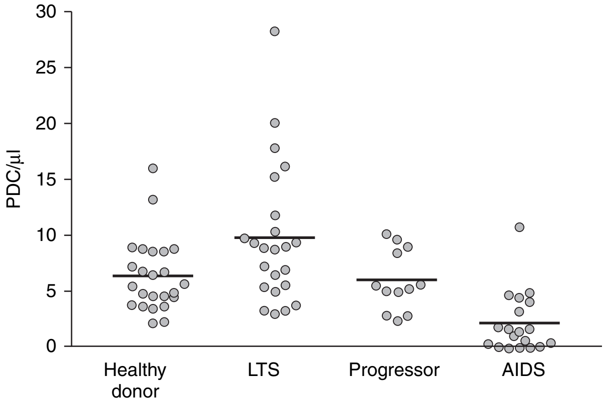

Figure 5 Relationship of plasmacytoid dendritic cell number to clinical state. Each open circle represents a value for a different study subject. Horizontal bars indicate the median. The number of blood (PDCs) is increased in long-term survivors (LTS) ($P <$ 0.05 for all group comparisons vs. LTS) and decreased in AIDS patients ($P < 0.01$) for all group comparisons vs. AIDS). Most of the progressors had received antiretroviral therapy for several months; no substantial difference in PDC number was observed between these subjects and those who were untreated. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

HIV infection may cause a decrease in dendritic cell number, impair their function as APCs, and decrease their ability to stimulate primary T cell proliferative responses and antibody production by B cells. A defect in DC renewal from CD34+ progenitor cells is also observed in HIV infection. PDC levels in HIV-infected individuals vary according to the clinical state (**Figure 6**). In advanced disease, the reduced PDC numbers correlate with decreased CD4+ cell numbers and high viral loads. LTS have higher levels of PDCs in blood compared with healthy controls. In acute infection, subjects with low viral loads have high PDCs compared with subjects with high viral loads. PDC numbers recover with early HAART but not after treatment interruption. Some untreated

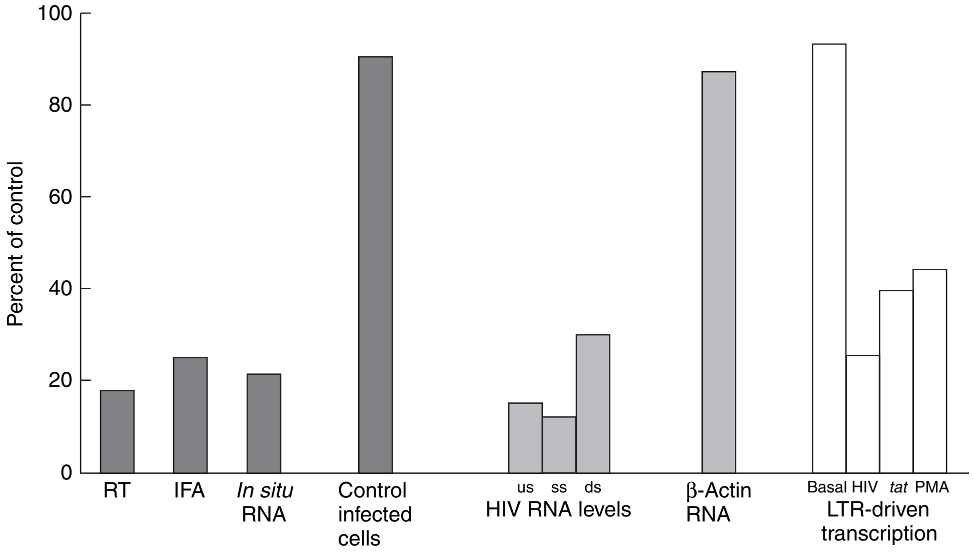

Figure 6 Effect of CD8+ cells and CAF on parameters of HIV replication. The CD8+ cell noncytotoxic antiviral response blocks viral replication, as indicated by decreased reverse transcriptase (RT) activity, viral protein expression measured by immunofluorescent antibody (IFA) techniques, and *in situ* RNA production. This activity has no effect on the number of infected cells in the culture. The antiviral effect is observed as well in a reduction in unspliced (us), single-spliced (ss), and double-spliced (ds) HIV RNA levels as compared to a normal expression of β-actin RNA. Finally, the suppressing effect of CD8+ cells or CAF does not affect the basal-level expression of HIV LTR-driven transcription but blocks induction of this transcription by HIV, SV-40 *tat*, or phorbol myristate acetate (PMA) using cells in which the HIV LTR has been linked to a reporter gene. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

asymptomatic HIV-infected subjects have normal or elevated PDC numbers even with low CD4+ cell counts.

HIV-infected cells induce the highest levels of type 1 IFN production by PDCs compared with HIV gp120 or free virus. The TLR involved in human PDC activation by HIV could be either TLR-9, or TLR-7, or both. After exposure to HIV-infected cells, PDCs mature as seen by their increased expression of CD80 and CD86. They also express CCR7, suggesting that they migrate to lymphoid tissues. This finding could explain their low numbers in HIV infection.

The production of IFN-α by PDCs in response to HIV-infected CD4+ cells has been shown to inhibit HIV replication in CD4+ cells. In addition, an increase in the CD8+ cell noncytotoxic antiviral response (CNAR) is observed when CD8+ cells from HIV-infected subjects are cocultured with PDCs or exposed to IFN-α.

The CD8+ cell noncytotoxic anti-HIV response (CNAR)

A subset of CD8+ cells can suppress HIV replication in infected CD4+ cells without killing the infected cells. HIV replication can be blocked at low CD8+ cell/CD4+ cell ratios ($<$ 0.05:1). This CNAR is somewhat stronger with cell activation and involves production of a CD8+ cell antiviral factor (CAF) (see below). CD8+ cells suppress viral replication in CD4+ lymphocytes and macrophage without affecting the proliferation or expression of activation markers on CD4+ cells and without killing the virus-infected cells. CNAR and CAF involve processes occurring after virus integration and prevent viral transcription (**Figure 6**). Some of the major characteristics of CNAR are detailed in **Table 8**.

CAF is a protein resistant to heat, low pH, ether, and lyophilization extraction processes. It lacks identity to any known protein or cytokine (e.g., α-defensins, β-chemokines) and is not found in cellular granules. CAF is produced in very low amounts by CD8+ cells (**Figure 7**) and its identification has not yet been achieved.

The CD8+ cell anti-HIV response develops soon after infection but diminishes over time, concomitant with progression to AIDS. It has been observed in healthy HIV-infected adults and children. In LTS this anti-HIV response is commonly found at high levels. In addition, highly exposed uninfected individuals usually have CNAR and often without evidence of cytotoxic T lymphocyte (CTL) activity. The antiviral CD8+ cells show this response against all HIV-1 and HIV-2 isolates tested.

CNAR is therefore different from the classic antigen-specific cytotoxic activity of CD8+ cells (see 'CD8+ T lymphocyte immune responses to HIV infection'). This CD8+ cell antiviral activity can also be observed with other lentiviruses such as SIV in various primate species and feline immunodeficiency virus (FIV)-infected cats. Since CNAR is not restricted by MHC, is mediated by a secreted cytokine, is not antigen-specific, and is an early and rapid response to HIV, it appears to be part of the innate immune system.

Table 8 Characteristics of the CD8+ cell noncytotoxic anti-HIV response (CNAR)

- Does not involve cell killing
- Property of CD8+ T cells; not CD4+ cells, NK cells, or macrophage
- Exhibited predominantly by the CD8+ HLA-DR+ CD28+ CD11b− human cell subset
- Associated with VCAM expression on CD8+ cells
- Blocks HIV replication in naturally or acutely infected CD4+ cells
- Can block HIV replication at low CD8+/CD4+ cell ratios (<0.05:1)
- Correlates directly with clinical status and high CD4+ cell counts
- Early response to HIV infection; occurs before seroconversion
- Active against all tested strains of HIV-1, HIV-2, and SIV
- Dose dependent
- Not MHC restricted
- Blocks HIV at transcription and does not affect earlier steps in virus replication cycle
- Observed with CD8+ cells from infected nonhuman primates
- Mediated (at least in part) by a novel soluble anti-HIV factor
- Optimal activity with cell–cell contact
- No effect on activation or proliferation of CD4+ cells

Measured by *in vitro* assays. MHC, major histocompatibility complex; NK cells, natural killer cells; SIV, simian immunodeficiency virus.

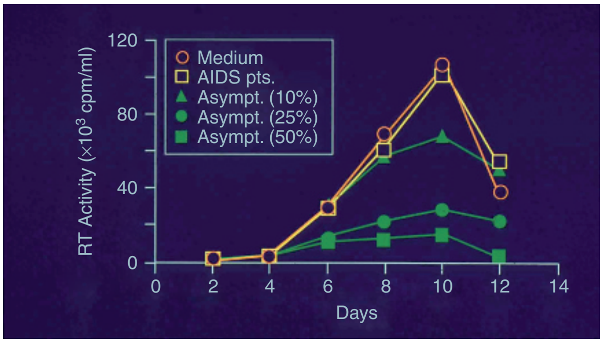

Figure 7 Quantity of CAF produced by CD8^{+} cells. Dilutions of CAF-containing fluid from cultured CD8+ cells from an asymptomatic individual indicate that a 1:4 dilution will still show a 50% reduction in HIV replication as measured by reverse transcription (RT) activity in the culture fluid. Fluids from CD8^{+} cells of normal individuals or those with AIDS do not show evidence of CAF production. Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

NK cells

NK cells are an important part of the immune system and represent 15% of the PBMC population. They recognize and in many cases kill virus-infected cells in a non-MHC-dependent manner. NK cells function through the interaction of cell surface inhibitory or activating molecules. Decreased expression of MHC class I molecules on virus-infected cells determines their susceptibility to NK cell killing. NK cell function is blocked by recognition of HLA-A, -B, and -C molecules by killer cell immunoglobulin-like receptors (KIRs), C-type lectins, and non-MHC class I molecules like CEA (CD66e). NK cell response against microbes includes production of immune regulatory cytokines (e.g., TNF-α and IFN-γ) as well as cytotoxic activity. NK cells can also eliminate HIV-infected cells through antibody-dependent cellular cytotoxicity (ADCC).

HIV infection causes a decrease in NK cell number and function. The reduced number correlates with decreased CD4+ cell counts. HIV viremia is associated with enhanced expression of NK cell inhibitory receptors and loss of activating receptors like NKG2A and CD94. HIV infection also causes impaired IFN-γ production, reduced ability to respond to IFN-α, and a decrease in ADCC mediated by NK cells.

Adaptive Immune Responses in HIV Infection

Humoral immune responses to HIV infection

Circulating anti-HIV antibodies can be detected in blood and mucosal surfaces. Antibodies appear usually within 1–2 weeks after acute infection. Gag is the first viral protein recognized followed by Nef, Rev, and Env. The IgG1 subclass is dominant in all clinical stages.

Anti-HIV neutralizing antibodies

Antibody-mediated neutralization of HIV is achieved when antibody binds with adequate avidity and appropriate specificity to the virus and inactivates (neutralizes) it. The viral envelope gp120 and gp41 glycoproteins are the primary proteins involved in antibody neutralization. The HIV-specific neutralizing antibodies are targeted to the V3 loop, the CD4-binding domain, and variable regions 1 and 2 of the envelope gp120. The viral envelope gp41, carbohydrate moieties, and other cell surface proteins on the envelope are also sensitive to antibody neutralization. Antibodies inhibit viral function in three ways: by inducing the dissociation of gp120 from gp41, by direct inhibition of viral binding to receptor–coreceptor complexes, and by interfering with postattachment steps of gp41 that lead to virus–membrane fusion.

Neutralizing antibodies against autologous virus are detectable within 4–8 weeks of primary infection. However, the virus isolated at the time the antibodies are detected is usually resistant to these antibodies. They neutralize only an earlier virus, not the concurrent virus that appears to escape this immune response. The anti-HIV neutralizing antibodies produced are present in low levels or absent in patients with progressive disease, but a strong and broad response is detectable in patients with long-term nonprogressive HIV disease. The neutralizing antibodies can also be important in preventing mother–child transmission.

A number of factors limit the development of effective HIV-specific neutralizing antibodies. These include the carbohydrate moieties, cell surface nonviral proteins like LFA-1, and the age of virus preparation in culture. Selective immunologic pressure allows the virus to escape neutralization by amino acid changes in the V3 loop and other envelope regions recognized by the neutralizing antibodies. This resistance to neutralization may be related to conformational masking of the envelope receptor-binding site on the virion. Various neutralizing immunotypes, not necessarily correlating with the genotype, are found involving a variety of clades.

Antibody-dependent cellular cytotoxicity

Antibodies to both gp120 and gp41 envelope proteins participate in ADCC-mediated killing of HIV-infected cells. In this process, antibody–antigen-coated cells are recognized by effector NK cells or by monocytes and macrophage bearing Fc receptors. They are killed either by perforin-mediated cytolysis or via apoptosis. The relative binding of these antibodies to the viral antigenic determinant depends on the specific gp120 and gp41 proteins expressed by different viral isolates. ADCC can have clinical relevance by destroying virus-infected cells, but it depends on the function of host effector cells like macrophage and NK cells. This activity has been detected in early stages of HIV infection and shown to be associated with a healthy state in LTS.

Detrimental effects of anti-HIV antibodies

Some antiviral antibodies can enhance viral replication through interaction with the complement or Fc receptor. This antibody-dependent enhancement (ADE) is associated with HIV disease progression. Complement-mediated ADE correlates with high plasma viral loads and is observed in patients with low CD4+ cell counts. Other harmful effects of antibodies include the presence of auto-antibodies that circulate in the blood of individuals with HIV infection. These auto antibodies can lead to several clinical conditions involving the loss of certain peripheral blood cells (e.g., neutropenia, thrombopenia) and to neuropathies.

T lymphocyte immune responses to HIV infection

CD4+ and CD8+ T lymphocytes exist as antigen-naive (naive) and antigen-experienced (memory) cells. The memory cells can further be broadly divided into two types. (1) Central memory T cells (Tcm) are those that circulate among secondary lymphoid tissue through blood and lymph channels. They express CCR7 and CD62L, which permits their trafficking to lymphoid tissue. (2) Effector memory T cells (Tem) are those that migrate from secondary lymphoid tissue into effector sites, like the intestinal lamina propria. They lack CCR7 and CD62L expression and remain at the site of microbial infection to respond to antigen. They can either become short-lived terminal effector cells or evolve into 'resting' memory cells with characteristics of central memory cells when the antigen load is reduced. The Tcm cells can rapidly produce cytokines and respond after restimulation by antigen exposure.

The CD8+ and CD4+ T lymphocytes recognize antigen processed to smaller peptides by APCs and presented at their cell surface in association with their MHC class I or -class II molecules, respectively. MHC class I molecules present proteolytic fragments (8–10 residues in length). This recognition of viral pathogens by CD8+ T cells is precise as the conserved residues of the $\alpha\beta$ heterodimer forming the peptide-binding groove bind to both terminal residues of the short peptides. In contrast, the peptide-binding groove of MHC class II molecules lacks these conserved residues and forms an open pocket, thus binding to a longer (12–24 residues) protein. This accounts for the diversity of antigenic determinants that can be presented to CD4+ T cells by MHC class II molecules.

The antigen is recognized by means of the $\alpha\beta$ TCR present on the T lymphocyte. Mature TCR genes are rearranged from multiple, discontinuous gene segments (V, D, J), resulting in a high level of diversification at the V(D)J junction (complementarity-determining region 3 or CDR3). The TCRs of various T cell clones responding to identical peptide–MHC complexes tend to exhibit structural similarities as the amino acid residues encoded within CDR3 closely contact the antigenic peptide. The sum of different TCR combinations and specificities within a host termed as the 'TCR repertoire' can determine the diversity and persistence of T cell immune responses. The diversity of the CD4+ and CD8+ T cell repertoires is also determined by the polymorphism of MHC class I and -class II genes, which is focused in regions of the molecules directly involved in peptide binding. A broad repertoire is associated with a beneficial clinical course. Thus, the repertoire alterations during HIV infection can result in either protective or deleterious immunologic responses.

A variety of assays can be used to detect CD4+ and CD8+ T cell responses to HIV. The intracellular cytokine assay, tetramer-binding assay, and CFSE cell proliferation assay are based on flow cytometry. Enzyme-linked immunospot assays utilize the principle of ELISA.

CD4+ T lymphocyte immune responses to HIV infection

The activated CD4+ T cells help orchestrate an effective immune response in HIV infection either through direct cell–cell interactions or through release of cytokines. The CD40L expressed on activated CD4 cells is crucial in triggering DCs via direct cell–cell interactions to produce IL-12, which in turn is central in initiating a CD8+ T cell response. T_H responses have also been divided into different subsets depending on the cytokine profiles of the stimulated cells:

- T_H1-type responses are associated with the production of type 1 cytokines (e.g., IL-2 and IFN-γ) that are supportive of cell-mediated immunity. These responses can be enhanced by cytokines like IL-12.
- T_H2-type responses are associated with type 2 cytokines (e.g., IL-4, IL-5, and IL-10) that promote humoral immunity and reduce CD8+ T cell responses.

Studies of HIV-infected individuals over time suggested that T_H1 (type 1) responses are present in healthy asymptomatic individuals, whereas T_H2 (type 2) responses predominate in the symptomatic phase of disease. A shift from type 1 to type 2 responses was found associated with disease progression. Thus, type 1 responses are thought to be protective. Although this hypothesis is less popular these days, it still contributes to the understanding of HIV disease progression.

CD4+ T cell responses to HIV have been divided into three cell subsets (primarily Tem) on basis of their ability to proliferate and produce cytokines: (1) those cells that secrete IL-2 and are most beneficial to host defense against HIV, (2) those cells that secrete IFN-γ and appear to be more differentiated with less proliferative and antigen-responsive activities, and (3) some cells that secrete both IL-2 and IFN-γ. Cytomegalovirus (CMV)-specific CD4+ cells and HIV-specific CD4+ cells in LTS have an equal distribution of all three subsets. However, HIV-infected individuals with progressive disease have solely IFN-γ-secreting CD4+ cells, which are considered terminal effector cells. HIV infection causes an increase in this subset as compared to CD4+ cells that secrete only IL-2 or IL-2 and IFN-γ and have good proliferation and antiviral response. The Tem cells that produce IFN-γ but not IL-2 also express CCR7. Recent studies show that disease-resistant sooty mangabeys have more IL-2-producing CD4+ T cells, thus underscoring the importance of this subset of CD4+ T cells in containment of HIV infection.

Strong HIV-specific CD4+ T_H responses have been observed in patients who are able to control viremia in the absence of antiviral therapy. In HIV-infected individuals with progressive disease, these responses are deleted or blunted as a direct or indirect consequence of ongoing viral replication. Several factors are thought to contribute to the impairment of HIV-specific CD4+ cell responses during progressive HIV disease. Besides direct cell death, the possible mechanisms include virus-induced anergy as well as antigen-induced cell death or apoptosis. HIV Tat downregulates HLA class II expression, and therefore impairs antigen recognition leading to virus-induced anergy. This process also prevents effective establishment of a memory cell population. Moreover, HIV-specific CD4+ cells are likely to be depleted because of direct HIV infection especially in acute infection.

Loss of effective CD4+ cell responses has detrimental consequences for HIV-specific CD8+ cell responses as CD4+ cell help is crucial for priming CD8+ cells, maintaining CD8+ cell memory, and maturation of functional CD8 cells. CD4+ cells may provide this help in two ways: (1) CD8+ cell activity is stimulated by IL-2 produced by CD4+ cells and (2) the upregulation of CD40L on CD4+ cells enhances the costimulatory pathways between APCs and CTLs. Moreover, CD40L triggers DCs to produce IL-12, which in turn initiates CD8+ cell responses. Several studies provide evidence that ineffective CTL responses are associated with lack of T_H cell responses. These observations underscore the importance of T_H cell activity in control of HIV infection. Memory CD8+ cells generated in the absence of CD4+ cell help are severely impaired in effector functions; they display poor recall responses and proliferation in comparison to memory CD8+ T cells generated with CD4+ cell help.

Certain CD4+ T cells, usually T_H1-type CD4+ T cells, can have CTL activity directed against either infected or uninfected CD4+ T cells or cells expressing HIV peptides in association with MHC class II molecules. This response is usually virus-strain specific, mediated by perforin or involves Fas–Fas ligand-induced apoptosis. Even though the level of CD4+ cell cytotoxic anti-HIV response is usually low, a substantial increase in HIV-specific CD4+ cell CTLs has been reported in primary HIV infection.

CD8+ T lymphocyte immune responses to HIV infection

The CD8+ T cells can have cytotoxic (CTL) or noncytotoxic anti-HIV activity (see 'Dendritic cells and HIV infection'). CD28 expression has been used to distinguish CD8+ T cells that have CTL activity (CD8+CD28–) and those with noncytotoxic anti-HIV activity (CD8+CD28+ cells). The HIV-specific CD8+CD28−CD57+CCR7− effector memory CTLs reflect chronic immune activation and have reduced proliferative capacity. This CD8+ T cell subset is increased in HIV infection. The cytotoxic effector memory CD8+ T cells also have a high expression of CD38 and HLA-DR, indicating their activated state.

HIV-specific CD8+ cytotoxic T cell responses are generated in the majority of infected subjects soon after the peak of viral replication during primary HIV infection. The result is a decline of plasma viremia, resolution of clinical symptoms, and a rapid selection of viral CTL escape variants. CNAR's role in this virus control has been shown (see 'Dendritic cells and HIV infection'). These cellular antiviral responses during acute infection involve more than 10% of total CD8+ cells and typically target, in a distinct hierarchical order, a small number of viral epitopes from the early expressed viral proteins, particularly Tat, Nef, and Rev. The responses are a result of a tightly regulated process that is critically impacted by the kinetics of viral protein expression, the genetic HLA class I background of the infecting individual, and the autologous sequence of the infecting virus. In LTS, HIV-specific CTL responses are directed against a wide range of HIV-1 antigens, including peptide determinants located within the Gag, Env, Pol, Rev, and Nef proteins. These responses persist at high frequencies; often 1–2% of all circulating CD8+ T cells are specific for a dominant HIV epitope. These responses are closely associated with the control of viral replication as demonstrated by their inverse correlation with viral load and loss of CD4+ T cells. HIV-1-specific CTLs can also suppress high virus replication in macrophage and kill infected macrophage.

However, HIV-specific CD8+ T cells become less effective in recognizing autologous virus over time. A decrease in HIV-specific CTL activity may occur in AIDS patients without a reduction in CD8+ cell cytotoxic functions to other pathogens. These HIV-specific CD8+ T cells can produce IFN-γ but have lower levels of perforin and persistent CD27 expression, which suggests impaired maturation and reduction in HIV-specific lytic activity. Thus, even though CTL activity correlates with a healthy clinical state, progression of disease with increasing numbers of HIV-infected cells, a high viremia, and low CD4+ T cell counts can occur in the presence of CD8+ effector cells.

CTLs recognize virus peptides presented by HLA class I molecules and different HLA types display different peptides. Thus, the quality of the immune response is affected. HLA types associated with slow progression of the infection such as HLA-B27 and HLA-B57 stimulate more effective immune responses compared with those that confer increased susceptibility such as HLA-B35. Similarly, homozygosity at the HLA class 1 loci, which is also associated with more rapid progression of HIV, offers less opportunity for a diverse T cell response.

HIV resistance to CTL activity can further compromise HIV-specific CD8+ T cell responses. The inability of CTL to eliminate HIV rapidly in primary infection exerts a selective force on the virus to have mutations in the dominant epitopes. This enables the infected cells to escape lysis and propagate the mutated virus. The immune escape by HIV may occur by any of the following mechanisms. (1) The infidelity of the viral RT results in the generation of viral mutations. Even a single mutation within a defined CTL epitope is sufficient to abrogate CTL recognition. (2) Viral latency, in which the level of expression of viral gene products is reduced, can prevent the presentation of viral peptides to the immune system. (3) Changes in the MHC class 1 molecule can lead to failure of HIV antigen presentation to immune system. Some viral gene proteins, especially Nef and Tat, downregulate HLA class 1 expression and impair CTL recognition. Moreover, the upregulation of the Fas ligand by Nef in infected cells can cause the HIV-specific T cells expressing Fas to be targets for killing by the FasL pathway. Other means by which HIV can escape CTL attack include sequestration of infected cells in the central nervous system where T cells normally have limited access.

Increased expression of the programmed death 1 (PD-1) protein on HIV-specific CD8+ T cells correlates directly with impaired CD8+ T cell function, increased viral load, low CD4+ cell counts, and progression to disease. The level of PD-1 expression is also associated with enhanced sensitivity of HIV-specific CD8+ T cells to apoptosis.

In summary, the CTL activity in response to HIV infection may appear to have beneficial effects but these cells can also have some detrimental effects. In some HIV-infected individuals, CTL can lyse autologous and heterologous activated, uninfected CD4+ lymphocytes, thus contributing to the loss of CD4+ T cells. The relative roles of CTLs and CNAR in HIV infection are under continual study.

Features of HIV Pathogenesis

Overview

HIV pathogenesis is a complex interplay between various biologic properties of the virus and the host immune response to the virus. The final outcome determines either long-term survival or progression to AIDS. As observed with HIV transmission, various cofactors appear to influence progression to disease. These cofactors include the genetic background of the host (e.g., HLA), infection by other viruses or pathogens, sexually transmitted diseases, immune-stimulating processes such as allergens, and factors that affect cytokine production. Certain lifestyle factors like smoking, alcohol, drugs, and stress can also influence HIV pathogenesis.

HIV pathogenesis can be divided into three major phases following acute virus infection (**Figure 8**).

Early period (Phase 1)

In the initial days following acute infection, virus replication takes place at the site of virus entry, particularly in activated CD4+ T cells, macrophage, DCs, and mucosal cells of the rectal and cervicovaginal cavities. Within 2 days, the virus goes from the initial site to the local lymph nodes, thus establishing infection. Viremia results with up to 5000 infectious particles per milliliter or 10^7 viral RNA molecules per milliliter of plasma detected in 5–7 days. After 10–14 days, approximately 200 billion CD4+ T cells are infected. This entire process depends on host susceptibility to infection and local immune factors.

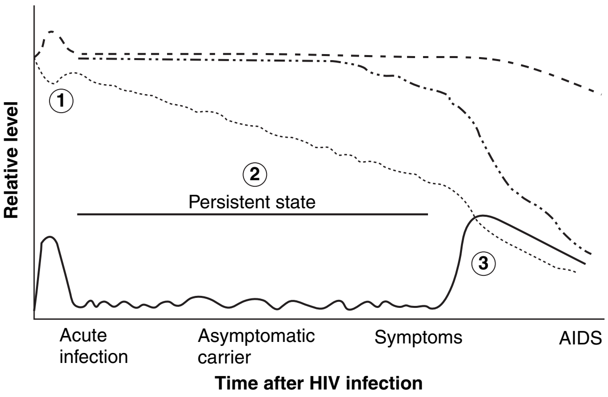

Figure 8 Events occurring after HIV infection. Prior to seroconversion, high levels of virus (- - -) can be detected in the blood. This viremia is then reduced to low levels (phase 1) and maintained with episodic release of varying amounts of virus over time (phase 2). A second high level of viremia occurs along with onset of clinical symptoms and remains raised throughout the development of AIDS (phase 3). The CD4+ cell number (. . . .) decreases during acute (primary) infection, then returns to a level somewhat below normal. A slow decrease in CD4+ cell count, estimated to be approximately 60 cells/μl per year, occurs over time (the persistent period) (phase 1). Subsequently, in some individuals developing symptoms, a marked decrease in CD4+ cell counts can be observed concomitant with reemergence of high levels of viremia (phase 2). The number of CD8+ cells (- - -) rises during primary infection, as is commonly seen in viral infections. Their number then returns to just above normal and stays elevated until the final stages of disease. In contrast, the CD8+ cell anti-HIV responses (..__..__) begin to decrease prior to or around the time of symptoms (late in phase 1) and then to decrease steadily as progression to disease occurs (phase 2). Reproduced from Levy JA (2007) HIV and the Pathogenesis of AIDS, 3rd edn. Washington, DC: ASM Press.

Subsequently, the CD8+ cell numbers rise as well as the production of proinflammatory cytokines and chemokines. The innate antiviral cytokines like type 1 IFNs and adaptive CD8+ T cell response do not appear at this stage to prevent infection. Thus, large numbers of target cells in lymphoid tissues become infected. The replicating virus can also undergo mutations to increase its virulence. Within weeks after acute infection, viremia levels decrease in association with the appearance of cellular immune responses, mainly CD8+ T cell responses with both cytotoxic and noncytotoxic activities. Seroconversion takes place within days to weeks after infection with transient appearance of neutralizing antibodies after about 2–3 months (see ‘Anti-HIV neutralizing antibodies’).

Persistent period (Phase 2)

The CD4+ T cell numbers usually return to their normal levels at 3–6 months after primary virus infection. However, their number steadily decreases either by direct cytotoxicity of the virus or by indirect means like apoptosis (see 'Overview: HIV cytopathology'). This gradual loss of CD4+ T cells can be observed in infected individuals from all risk groups. During this phase, HIV replication in the body continues at a low level. This suppression of HIV replication appears to be mediated by antiviral CD8+ T cells. The virus population becomes heterogeneous, reflecting the ongoing emergence of virus variants that are able to escape the immune responses.

Symptomatic period (Phase 3)

Many infected individuals develop symptoms within 8–10 years after infection. The CD4+ T cell counts drop below 350 (sometimes 200) cells μl^{-1}. The viral load increases with a reduction in antiviral CD8+ T cell immune responses. The reduced cellular immune responses also lead to increased viral replication in the lymph nodes and disruption of lymphoid tissue including follicular DCs. The high levels of virus in blood and lymph nodes are accompanied by the emergence of a predominant virus strain as observed in primary infection. However, this virus strain is usually virulent as represented by the X4 phenotype compared with the noncytopathic R5 phenotype in primary infection. Some of the characteristics of the virulent variants include extended cellular host range, rapid kinetics of replication, high levels of syncytia induction, increased CD4+ cell cytopathicity, resistance to neutralization, sensitive to antibody-mediated enhancement of infection, and failure to enter into a latent viral state. The virus responsible can be a R5 biotype in 50% of cases, but it has biologic properties of virulence including cytopathicity and increased replicative ability.

A loss of anti-HIV immune responses further enhances the disease progression and onset of AIDS. The various factors involved in the decrease of HIV-specific immune function have been detailed in the section titled 'Host immune responses to HIV'. These include chiefly the loss of CD4+ T cells by direct killing effects of virus or by indirect effects such as apoptosis, inadequate production of type 1 cytokines (IL-2), reduced cell production in the thymus, and increased production of type 2 cytokines (e.g., IL-10), which can further reduce CD8+ T cell-mediated immune responses and cause aberrant immune responses like autoreactive T cells.

Factors Affecting HIV Disease Progression

The CD4+ T cell counts and viral RNA levels are the most important predictors of prognosis in HIV infection. In the absence of therapy, a delay in progression to disease correlates with low viral RNA levels and high numbers of CD4+ T cells in the blood. In this regard, baseline viral loads of >100 000 RNA copies ml^{-1} and CD4+ T cell counts <350 cells μl^{-1} are indicators for initiation of antiretroviral therapy. A poor prognosis is also associated with high plasma levels of certain markers of immune activation like neopterin, tumor necrosis factor, IL-2 receptor, sCD8, sCD4 proteins, and $\beta 2$ microglobulin. Increased expression of CD38 on CD8+ T cells also reflects immune activation and directly correlates with poor prognosis. These markers can be particularly important in individuals with discordance in viral loads and CD4+ T cell counts. Other prognostic factors indicating disease advancement include increased levels of p24 antigen or infectious virus in the blood, low titers of antibodies to the HIV p24 or p17 Gag and the Nef proteins, low serum albumin, and decreased delayed-type hypersensitivity reactions.

Some of the virus characteristics found with disease progression have been discussed in the sections titled 'HIV infection and replication' and 'Effect of HIV on cells and on the immune system'. X4 virus infection is associated more often with a fast disease progression than R5 virus infection. However, R5 viruses can still account for 50% of AIDS cases. As noted above, these R5 viruses have greater replicative abilities than those from HIV-infected asymptomatic individuals. In some cases, a deletion in the viral *nef* gene or a mutation in the *vpr* gene is associated with slow disease progression. Moreover, individuals infected with clade C, -D, or -G viruses are more likely to develop AIDS than people infected with clade A or -B viruses.

HIV disease progression also depends on certain host factors, such as some HLA genotypes (see 'T lymphocyte immune responses to HIV infection'). Polymorphisms in certain chemokine receptors also affect the pathogenic pathway. For example, individuals homozygous for the CCR5 $\Delta 32$ allele (deletion in expression of CCR5) show resistance to infection by R5 viruses. Those with a CCR5−/CCR5+ genotype can have a delayed progression to AIDS. A haplotype of the CXCR1 gene (CXCR1-ha), one of the receptors for IL-8, may have a protective role in disease progression. Single-nucleotide polymorphism (SNP) studies of host immune factors like RANTES, SDF, IL-4, and the DC-SIGN promoter have recently shown that RANTES-28G is associated with late AIDS progression whereas DC-SIGN-139C is linked with accelerated clinical course in HIV-1-infected Japanese patients suffering from hemophilia. Modifications in certain chemokines and the age, gender, race, and ethnicity of a population can determine HIV disease progression.

Clinical Outcome of HIV Infection

The major clinical outcomes of HIV infection can be distinguished:

1. Typical progressors consist of individuals who progress to disease 8–10 years after infection. Their immune functions appear to be intact in early infection but gradually decline.

2. Rapid progessors show a very quick decline in CD4+ T cell counts, usually within 2–5 years of infection. They are characterized by high viral load and viral homogeneity. They have low levels of anti-HIV-1-neutralizing antibodies. CTL activity may be present in these individuals, but is functionally impaired as reflected by high viral load. They also have an activated immune state represented by large numbers of CD38 and HLA-DR-expressing CD8+ T cells.
3. Long-term survivors (LTS) or long-term nonprogessors (LTNP) are HIV-infected individuals who have remained asymptomatic with normal CD4+ T cell counts and low viral loads in the absence of therapy for at least 10 years after infection. The rate of CD4+ decline is markedly reduced in LTS compared with normal progressors. The usual percentage of LTS is 5–8% of total infected people. A subset of LTS, sometimes called elite controllers, remain healthy for many years with very low or no detectable virus in their blood.
4. Factors involved in long-term survival can include infection with a less cytopathic R5 strain and a beneficial anti-HIV immune response reflected by the presence of neutralizing antibodies and a lack of enhancing antibodies, the presence of anti-HIV CTL, and certain innate immune responses such as CNAR and high levels of interferon-producing cells or PDCs. PBMCs from LTS also show a type 1 cytokine (IL-2 and IFN-γ) production favoring CD8+ T cell immune responses. The genetic background of the host like HLA and chemokine receptor polymorphisms also influence long-term survival.
5. High-risk seronegative individuals have been exposed on many occasions to HIV but remain uninfected. This group includes sexual partners of infected individuals, intravenous drug users, transfusion recipients, patients suffering from hemophilia, children born to infected mothers, and healthcare workers with needlestick injuries. They have a reduced or lack of CCR5 expression and genes closely related to HLA class A2 alleles. These individuals can have HIV-specific CD4+ and CD8+ T cell immune responses as well as CNAR but no antiviral antibodies. In some cases, they have a reduced state of immune activation and decreased susceptibility of their PBMCs to infection. Increased NK cell function associated with a more effective response immune response to HIV and high RANTES levels have also been observed in some people in this group. Recent studies have also shown an increased expression of APOBEC3G in PBMCs of high-risk seronegative individuals (see 'Differences in virus production'). Polymorphism in the IRF-1 gene has been found as well to be associated with resistance to HIV infection in these individuals.

Future Directions

Combination antiretroviral therapy or HAART has changed the course of HIV infection by enabling HIV-infected people to survive longer. Many drugs that target certain features of HIV infection, particularly steps in viral infection, have been evaluated. To date, more than 20 drugs are being used in various combinations. Broadly, the drugs can be categorized as attacking the viral enzymes, RT, protease, and most recently, integrase. Some novel therapies under consideration include the use of RNA interference, which targets specific viral genes, and drugs that block viral activity via the chemokine coreceptors or virus fusion (e.g., Fuzeon) occurring after virus attachment to cells.

While HAART has been able to control HIV infection in individuals who are even suffering from AIDS, the long-term control of this virus requires new directions. Besides consideration of latent infections, the emergence of recombinant viruses and drug-resistant strains as well as the toxic effects of HAART challenge therapies and can further limit the benefits of antiretroviral strategies. Importantly, the virus-infected cell still remains a mechanism for HIV transfer, since antiretroviral drugs do not directly eliminate this source of the virus.

Further work therefore needs to be done toward boosting the immune response via both the innate and the adaptive immune systems and the development of an effective vaccine. Enhancement of innate immune responses is particularly important at mucosal sites of HIV transmission. One emphasis for future therapies is to restore the immune system to that of LTS who can control HIV infection. This approach would include a variety of cytokines, including IL-2, IL-15, IFN-α, IL-7, and, when fully identified, CAF. In vaccine development, these same cytokines can act as beneficial adjuvants along with CpG, G-CSF, and Flt-3 that help elicit innate immune responses by PDCs. An innovative way of excising HIV-1 proviral DNA from cells using an evolved recombinase system has been recently demonstrated. Improvements in these kinds of approaches could also provide the basis for future therapies to control HIV.

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Horizontal Gene Transfer: Uptake of Extracellular DNA by Bacteria

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Defining Statement
Process of Natural Uptake of Extracellular DNA in Bacteria
Uptake of Extracellular DNA by Bacteria Present in Various Environments
Some Limitations of the DNA Uptake Model Systems Used
Factors Affecting the Stable Uptake of DNA in Single Bacterial Cells
Considerations of the Long-Term Persistence of Horizontally Acquired DNA in Bacterial Populations
Predictors of DNA Uptake in Bacteria and Implications for the Release of Genetically Modified Microbes
Further Reading

Glossary

extracellular DNA Extracellular DNA (also called naked, free, ambient, or environmental DNA) are DNA fragments released into the environment from decomposing cells, disrupted cells, or viral particles, or via excretion from living cells.

heterogamic/interspecific transformation Heterogamic/interspecific transformation leads to recombination between chromosomal DNA from different species.

homogamic/intraspecific transformation Homogamic/intraspecific transformation leads to recombination between chromosomal DNA within species.

recipient Recipient is a bacterial cell that has the potential to acquire DNA by natural transformation.

synteny Synteny describes the observation of shared presence and order of genes on the chromosomes of related species. Disruption of synteny due to genetic rearrangements may reduce recombination rates within and between the species.

transformant Transformant is a bacterial cell that has successfully acquired DNA by natural transformation.

transformation frequency Transformation frequency is usually given as the number of transformant bacteria divided to the total number of recipient bacteria of a given species over a given time period.

Abbreviations

CDS	coding sequence
GMM	genetically modified microbes
GMO	genetically modified organism
HFIR	homology-facilitated illegitimate recombination
HGT	horizontal gene transfer
MEPS	minimum efficient processing segment
MMR	methyl-directed mismatch repair
RM	restriction modification
SSBP	single-strand binding proteins
USS	uptake signal sequences

Defining Statement

Here we present experimental models that examine the uptake of chromosomal DNA in bacteria and discuss some of the advantages and limitations of the models used. The relationship between DNA uptake frequencies versus selection is examined, and the implications for the open release of genetically engineered microbes is discussed.

Process of Natural Uptake of Extracellular DNA in Bacteria

Extracellular DNA released from a donor organism can be ‘horizontally’ acquired by bacteria (recipients) through the process of natural transformation. Natural transformation occurring with chromosomal DNA fragments can be divided into several steps:

1. development of competence in the recipient bacterium simultaneously with exposure to transforming DNA;
2. DNA uptake/translocation into the bacterial cytoplasm;
3. heteroduplex formation of transforming DNA with similar DNA sequences in the recipient chromosome;
4. homologous recombination-mediated integration of the transforming DNA strand into the recipient bacterium's chromosome;
5. expression of the acquired trait(s) leading to an altered recipient phenotype; and
6. stable inheritance and maintenance of the acquired trait(s) at the individual recipient cell level and at the bacterial population scale level.

Although the last two steps are not strictly part of the transformation process, they are usually an integral part of most natural transformation studies in bacteria. Extracellular DNA is only accessible to naturally transformable bacteria when they are in a competent state. Competence is a genetically encoded physiological state in which bacteria express protein complexes that facilitate DNA uptake into their cytoplasm. In bacteria such as *Streptococcus pneumoniae* and *Bacillus subtilis*, competence is under tight regulation by a cell-to-cell signaling pathway. For other bacteria, such as *Neisseria gonorrhoea*, competence is constitutive and DNA can be taken up during all phases of growth. Competence development is usually linked to particular growth conditions or perturbation of those conditions. For instance, *Acinetobacter baylyi*, a soil and water bacterium, achieves maximum competence for DNA uptake during early exponential phase, with competence peaking at approximately midexponential phase. In *B. subtilis*, random peak levels of the ComK protein trigger transition to a competent stage. Bacteria that can express natural genetic competence are found in diverse habitats, and it is suspected that discovery of more naturally competent species and strains will continue with further improvement of culture- and nonculture-based methods of detection of DNA uptake. The reasons for the existence of DNA uptake/integration mechanisms in bacteria are still debated; incoming DNA fragments may be used for nutrition after further degradation, as a template for recombinational repair of DNA, or for the generation of genetic variation of which some may accelerate adaptation. Natural transformation may serve different functions in different bacterial species, as evidenced by expression of competence at dissimilar stages in their life cycles and to varying extents.

The model organisms *B. subtilis*, *S. pneumoniae*, *A. baylyi*, *N. gonorrhoea*, and *Haemophilus influenzae* have provided most of the information about the molecular aspects of the natural transformation process. The DNA translocation apparatus is a putative pore-forming multimeric protein complex that spans the inner membrane/periplasm/outer membrane in Gram-negative bacteria, and the inner membrane/cell wall in Gram-positive bacteria. Extracellular DNA first binds to the translocation apparatus on the cell surface by a poorly understood mechanism. While *N. gonorrhoea* and *H. influenzae* require specific uptake signal sequences (USS) for successful DNA binding and translocation, the majority of bacteria will bind extracellular DNA nondiscriminately with respect to sequence. Both linear and circular DNA are taken up during natural transformation. Structural proteins associated with extracellular DNA do not hinder the DNA-binding or uptake process, as bacterial cell lysates also efficiently transform competent bacteria. In most cases, although the DNA is initially double-stranded, it enters the bacterial cytoplasm in single-stranded (ssDNA) form, providing evidence for an endonuclease within, or associated with, the membrane-bound translocation apparatus that degrades one strand during DNA uptake. The uptake of DNA by competent cells occurs at rates of up to $100\,\mathrm{bp\,s^{-1}}$. Studies on the size of chromosomal DNA substrates in bacteria have demonstrated that the initial DNA fragment size is not correlated to uptake efficiency but to integration efficiency. Longer DNA fragments (5 kb or more) are more likely to yield detectable transformants in *in vitro* assays than shorter fragments. In some cases, part of the translocated DNA can be degraded in the cytoplasm (e.g., approximately 500 bp at each end of the linearized strand) prior to chromosomal integration.

Few details are available on the molecular interactions of ssDNA once it enters the cytoplasm of studied bacterial species. There is some evidence that single-strand binding proteins (SSBP) or other proteins bind to the DNA fragment and provide protection from rapid nucleolytic degradation. Although the majority of DNA molecules that enter the bacterial cytoplasm will be degraded, some of the ssDNA may subsequently pair with the bacterial chromosome by homology-guided base-pairing at the cognate locus in the recipient. The resulting duplex molecule acts as a substrate for resolution by the RecA protein. For heterogamic transformation (i.e., recombination between chromosomal DNA from divergent species), the formation and stability of the heteroduplex molecule formed by donor strand base-pairing is determined by the degree of sequence similarity between the donor and recipient DNA. If the incoming chromosomal DNA is less than 30% divergent from the recipient genome, integration by homologous recombination may occur into the host genome. Natural transformation with chromosomal DNA in bacteria seems to depend strongly on DNA sequence similarity. Lack of DNA similarity and, hence, stable heteroduplex formation is, for example, the most likely hindrance to interspecific recombination between *Bacillus* spp., *Streptococcus* spp., and *Acinetobacter* spp.

The most common type of recombination in bacteria is thought to occur when sequence similarity between the donor and recipient DNA is uniformly present over the entire heteroduplex region. In cases where the donor and recipient sequences are identical along their entire lengths, recombination with the donor DNA strand will not result in a detectable genetic or phenotypic change. In cases where some minor sequence dissimilarity between donor and recipient result exists, successful transformation events yield the acquisition of donor polymorphisms in transformants (i.e., allelic replacement). This process is called substitutive recombination, as the resulting DNA sequence in a transformant originates by substitution of the recipient sequence with (parts of) the donor sequence. Such recombination events do not introduce major insertions or deletions and typically do not disrupt the overall coding sequence (CDS) and reading frame if a protein-encoding gene is present in the recombined region.

A second type of recombination process occurring in natural transformation is called additive integration. Such a recombination process occurs when a defined DNA sequence present only in the donor is flanked on both sides by sequences common to both donor and recipient bacteria. Heteroduplex formation occurring at flanking regions with high DNA similarity results in recombination of those sequences, as well as integration of the intervening foreign sequence, into the recipient chromosome. Additive integration may also occur in the presence of only one-sided homology/DNA similarity between donor and recipient. Such homology-facilitated illegitimate recombination (HFIR) allows additive integration of an incongruous DNA sequence via a recombinational anchor of homologous/similar sequence at one end of the invading DNA strand and a random microhomology (3–12 nucleotides) at the other end. HFIR events often result in nonspecific deletion of a recipient DNA sequence located between the anchor and the downstream microhomology. HFIR has been demonstrated as a mechanism of foreign DNA integration by natural transformation in *A. baylyi*, *S. pneumoniae*, and *Pseudomonas stutzeri*. Where DNA integration is additive, the activity of the DNA maintenance machinery, combined with mutations and selection of the host chromosome, may over time result in the nucleotide composition of the integrated fragment resembling that of the recipient. Also, a gradual elimination of nonpositively selected DNA may occur by processes not fully understood.

A third type of recombination, illegitimate recombination, could hypothetically occur also in the absence of DNA sequence similarity (e.g., <70% DNA similarity) between the donor and the recipient bacteria. However, in contrast to many eukaryotic systems, illegitimate recombination of nonmobile chromosomal DNA in competent bacteria has been rarely reported. Random insertion of foreign DNA in bacterial genomes most often results in a reduction in the relative fitness of the transformant. It can, therefore, be hypothesized that such events are extremely rare in bacterial populations and the forces modulating recombination frequencies in bacteria are complex (**Figure 1**).

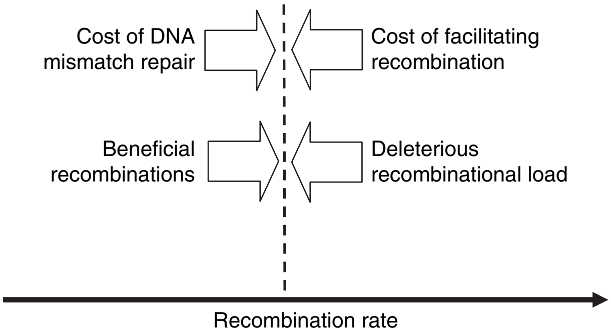
Cost of DNA mismatch repair
Cost of facilitating recombination
Beneficial recombinations
Deleterious recombinational load
Recombination rate

Figure 1 Recombination frequencies may vary according to the metabolic (fitness) costs and benefits for the transformant. Recombination events can increase transformant fitness via rare acquisition of novel beneficial traits. Recombination rates are kept low by the high deleterious recombinational load associated with accumulation of deleterious sequences and the costs associated with production and maintenance of DNA translocation/recombination machinery. Recombination rates may be raised by the metabolic costs associated with frequent removal of unsuccessful recombination intermediates by DNA mismatch repair.

Natural transformation with DNA sequences encoding their own mobility and stabilization functions (e.g., mobile DNA such as plasmids) may occur independent of recombination with the bacterial chromosome as these can recircularize and replicate in the bacterial cytoplasm. The stable uptake of plasmid DNA in competent bacteria usually occurs at lower frequencies than the integration of chromosomal DNA fragments due to reassembly constraints on the plasmid fragments in the cytoplasm.

The resulting recombinant locus in the transformant bacterium may contain genetic alterations in existing noncoding (e.g., regulatory) or protein-coding sequence, or larger alterations such as changed gene composition, gene order (synteny), and so on. The various combinations of these factors may result in favorable, unfavorable, or near-neutral fitness changes to the transformed bacterium that will ultimately determine the impact and the survival of the transformant in a dynamic bacterial population over time.

Uptake of Extracellular DNA by Bacteria Present in Various Environments

Extracellular DNA molecules are released into the environment from decomposing cells, disrupted cells, or viral particles, or via excretion from living cells. Release of intact DNA from decomposing cells depends on the activity and location of intracellular nucleases and reactive chemicals. DNA released from dead cells will be usually associated with the constituents of the cell cytoplasm, membrane, and DNA-binding proteins.

The first indication of the capacity of bacteria to take up extracellular DNA was published in 1928 by Griffith, when avirulent nonencapsulated Streptococci in mice were 'transformed' into the virulent, capsulated form after exposure to a capsulation 'factor' from heat-killed virulent Streptococci. The experiments of Avery, MacLeod, and McCarty in 1944 identified deoxyribonucleic acid (DNA) as the transforming factor in Griffith's experiments. Natural transformation studies of *B. subtilis* and *H. influenzae* followed during the 1960s. Researchers then expanded studies to investigate interspecific genetic exchange in *Streptococcus* spp., between *H. influenzae* and *Haemophilus parainfluenzae*, and between different species of *Bacillus*. The ability to take up extracellular DNA by natural transformation has since been detected in a range of divergent subdivisions of bacteria, including representatives of the Gram-positive, cyanobacteria, *Thermus*, *Deinococcus*, Green-sulfur bacteria, and numerous Gram-negatives. Recent studies have shown that some archaea and the single-celled eukaryote *Saccharomyces cerevisiae* (Baker's yeast) can develop competence for natural transformation as well. The phylogenetically widespread ability to acquire genetic information by natural transformation suggests that it may be functionally important in the environment. The limited number of transformable species identified may be explained by an overall lack of competence in the test populations, inability to obtain sufficient testable population sizes and time scales in the laboratory, or inability to recreate competence-inducing conditions as they occur under natural conditions.

Uptake of DNA by Soil Bacteria

A number of divergent bacterial species present in soil, including *A. baylyi*, *B. subtilis*, *P. stutzeri*, and *Thermoactinomyces vulgaris* are known to be naturally transformable. So far, all the published studies with soil-derived bacteria have been conducted in laboratory microcosms with sterile or nonsterile soil samples. Often, the soils have been amended with clay minerals or nutrients prior to or during the transformation experiments. Natural transformation of bacteria in the field remains to be shown, possibly due to the experimental challenges in both identifying and quantifying low-frequency events occurring in bacterial communities reaching 10^9 bacterial cells per gram of soil. Some of the first transformation studies on soil reported that the commonly occurring Gram-positive bacterium *B. subtilis* takes up chromosomal DNA when grown in autoclaved potting soil. Many subsequent studies have investigated natural transformation of *B. subtilis* and *Bacillus amyloliquefaciens* cells in soil with chromosomal and plasmid DNA. Often, the addition of clay minerals (1–10%, w/w) such as montmorillonite has been shown to increase DNA stability and transformation frequencies. Natural transformation of *P. stutzeri* cells has been shown in sterile soil slurry with chromosomal DNA, and in a nonsterile soil microcosm with plasmids, chromosomal DNA, and intact bacterial cells. While most studies in soil microcosms have relied on the addition of purified DNA, natural transformation of the Gram-negative *A. baylyi* has also been shown after the addition of isogenic cell lysates or lysates from *Pseudomonas fluorescens* or *Burkholderia cepacia*. The detection of DNA uptake was based on recombinational repair of a partially deleted neomycin phosphotransferase gene (*npt*II) in the recipient with a functional *npt*II gene transferred from the donor bacteria. While most studies of natural transformation in soil have relied on the addition of competent cells to the microcosms, some studies have also shown that competence for natural transformation can develop *in situ*.

Several investigations have explored the possibility that plant transgenes, such as antibiotic resistance markers, can be released from decaying genetically modified plants and be exposed to competent soil- and plant-associated bacteria. Short fragments of plant DNA have been shown to remain stable for up to several years in agricultural soil. Soil microcosms have been used to investigate the possible transfer of the kanamycin and hygromycin resistance genes (*npt*II, *hpt*) from tissue homogenates of transgenic tobacco (*Nicotiana tabacum*) plants into indigenous soil bacteria. However, no bacterial transformants could be recovered. This investigation hypothesized that any integration of the plant marker genes into the genome of exposed soil bacteria would take place after illegitimate recombination. The only study that has shown uptake of DNA fragments isolated from transgenic plants into a soil-residing bacterium utilized a genetically engineered strain of *A. baylyi* with inserted sequence similarity to the plant marker gene *npt*II. Using this system, the uptake of the plant marker gene in the bacterium was shown in a sterile soil microcosm at frequencies of 1×10^{-7} transformants per plant-harbored copy of the *npt*II gene. The amount of DNA added was several orders of magnitude higher than the concentrations expected to be released from plants under their natural growth cycle. There is no evidence to date for stable incorporation and inheritance of plant DNA in bacteria in the absence of supplied DNA sequence similarity.

Soil is a spatially and structurally heterogeneous environment in which numerous microhabitats exist. The current empirical knowledge of natural transformation in soil has been exclusively collected in soil microcosms. Most often, the studies have relied on external addition of high concentrations of DNA, bacterial cells, nutrients, or clay minerals to either sterile soil (all studies prior to 1997) or nonsterile soil samples. Moreover, the transformation frequencies recorded in soil represent so far a broad distribution across a heterogeneous milieu. The detection of rare natural transformation events under more realistic conditions depends on advances in methodology to allow more sensitive identification and quantification in the locations in soil that are conducive to natural transformation.

Uptake of DNA by Plant-Associated Bacteria

Both transduction and conjugation have been shown to occur on plant surfaces. However, few studies have examined horizontal gene transfer by natural transformation on plant surfaces or plant tissues. A growing interest in gene transfer mechanisms active in these environments has been stimulated by the presence of engineered genes (transgenes) in genetically modified plants. Candidates for exposure to plant transgenes are bacteria that interact closely with plant cells, for example, epiphytes, endophytes, rhizosphere bacteria, and plant pathogens. Several studies have examined the potential of DNA uptake in plant-associated bacteria. Uptake of plant marker genes has been examined in the plant pathogen *Agrobacterium tumefaciens* in tobacco crown-galls, in transgenic potato tubers infected with a pathogenic *Erwinia chrysanthemi* strain, and *Ralstonia solanacearum*-infected tomato plants. A range of studies has been performed with the soil bacterium *A. baylyi*. So far, all attempts to detect horizontal transfer of plant transgenes into *naturally* occurring plant pathogenic bacteria have been unsuccessful. The general 1000-fold larger size of a plant genome, as compared with a bacterial genome, effectively dilutes the concentration of the selectable gene examined for transfer. Transgenes inserted into plant organelles may have higher copy numbers and higher sequence similarity to bacterial chromosomes than transgenes inserted into the plant chromosomes. Some recent studies have shown that bacteria can access plant DNA during colonization of plants, or after mechanical disruption of plant tissues, if regions of high DNA similarity exist between the plant and bacterial DNA. Thus, the main mechanistic barrier to the uptake and integration of plant transgenes in bacteria seems to be the limited ability of plant transgenes to act as a substrate for heteroduplex formation and homologous recombination.

Uptake of DNA by Bacteria in Water and Sediment

Marine environments can contain significant concentrations of dissolved DNA. Several studies have examined the ability of both introduced and native bacteria to take up DNA by natural transformation in water and sediment microcosms. The studies can be divided into those that introduce defined bacterial inoculants (recipients) or purified selectable DNA (e.g., containing an antibiotic resistance marker gene) into marine microcosm environments to detect DNA uptake events, and those that expose selected members of the indigenous population to DNA containing selectable markers *in vitro*. In studies utilizing introduced bacterial recipients, natural transformation has been shown in the *Vibrio* spp. strain WJT-1C (later reclassified as a pseudomonad) with plasmid DNA in small-scale marine water and sediment microcosms sampled from the Eastern Gulf of Mexico; in marine sediments inoculated with the *P. stutzeri* and added chromosomal DNA; in *Acinetobacter calcoaceticus* strain BD413 (recently renamed *A. baylyi*) with chromosomal DNA in groundwater samples from a drinking water well, sterile aquifer material, and in sterile groundwater microcosms; and in *B. subtilis* with chromosomal and plasmid DNA bound to mineral aquifer material.

Escherichia coli K-12 strains have been found to develop competence and take up naked DNA (pUC18) in water samples taken from calcareous regions. The high Ca^{2+} concentrations found in the mineral springs produced up to 20 000 transformants of *E. coli* per 10^8 strain JM109 cells. In the first experiments to describe natural transformation in open systems, an introduced *A. baylyi* strain was capable of being transformed to prototrophy by bacterial cell lysates or live donor cells in different river systems. The recipient *A. baylyi* bacteria and transforming DNA were immobilized on filters secured to stones on the riverbed. Transformation frequencies of 10^{-6}–10^{-3} were reported.

In native bacterial populations, natural transformation of marine bacterial populations has been recorded after exposure to plasmid DNA or DNA in cell lysates of rifampicin-resistant *Vibrio* strains. Three out of 30 marine bacterial isolates were found to be competent for uptake of the plasmid DNA in *in vitro* assays. These included isolates of the genera *Vibrio* and *Pseudomonas*. Moreover, 15 out of 105 sensitive isolates obtained from Tampa Bay (FL, USA) were found to acquire rifampicin resistance in *in vitro* assays. In another set of experiments, plasmid DNA exposure of 14 different whole bacterial communities sampled from a variety of marine environments, such as sediment, surface, and deep water, and from various organisms revealed that bacteria present in 5 out of the 14 communities examined could take up DNA. This study estimated that between 0.000 05 to 1 transformant occurred per liter of water per day, suggesting that natural transformation may be an important mechanism for plasmid transfer among marine bacterial communities. In general, experimental studies suggest that DNA present in freshwater, in marine water, and on sediment surfaces is available for natural transformation of competent bacteria of the genera *Acinetobacter, Bacillus, Pseudomonas*, and *Vibrio*, albeit at variable frequencies that depend on environmental conditions and the type of DNA present. The introduction of defined recipient bacteria into an environmental sample results in competition between the introduced recipient population and indigenous communities for nutrients, habitats, and transforming DNA. This presents a challenge in designing experiments that accurately reflect the environmental conditions, species compositions, and concentrations encountered by recipient bacterial strains when present in natural habitats.

Uptake of DNA by Bacteria Present in the Digestive System

The animal digestive system is hypothesized to be an environmental hot spot for bacterial gene transfer due to the high concentrations of nutrients, actively growing bacteria, and surface. However, due to methodological constraints and other undetermined factors, little information is available on DNA uptake processes active in the digestive system of animals. The potential for competence has been identified in few bacteria isolated from this system. Most studies have focused on members of the Gram-positive genus *Streptococcus* that are ubiquitous in the oral cavity and rumen of many higher animals including cattle and sheep. Several microcosms and *in vitro* systems have been applied to detect DNA uptake in *Streptococcus* spp.

Sampling the teeth of 70 human individuals, it was found that at least 20 out of 129 isolates identified as *Streptococcus mutans*, which forms biofilms in dental plaques, could develop competence for acquisition of chromosomally borne streptomycin resistance *in vitro*. In a more recent study, high natural transformation frequencies were reported, reaching up to three transformants per 100 exposed recipients, using *S. mutans* cells grown in biofilms on polystyrene microtiter plates and saturating concentrations of DNA. The transformation frequencies of the surface-bound cells were 10- to 600-fold higher than those observed for planktonic *S. mutans* cells. Natural transformation was also observed when *S. mutans* cells were exposed to biofilms of a heat-killed isogenic strain harboring an erythromycin resistance gene. Natural transformation of *Streptococcus gordonii*, also found in the human oral cavity, has been reported by plasmid or chromosomal DNA in saliva samples at frequencies up to 7×10^{-4} transformants per recipient cell. DNA released from bacteria or food sources within the mouth can, therefore, be potentially taken up by naturally transformable bacteria. The natural transformation of a ruminal bacterium was first reported in 1999 with *Streptococcus bovis* cells at a frequency of 1×10^{-5} transformants per microgram plasmid in a liquid culture medium.

Currently, only a few bacterial species localized to the digestive system of higher animals have been found to express competence *in vitro* and none have been found *in situ*. Few studies have examined or reported the potential development of competence by bacteria colonizing invertebrates. The scarcity of available data may be due to insufficient incentive and methods to investigate limited bacterial transformability in such habitats. Although several members of the genus *Streptococcus* have been found to develop competence *in situ*, and other bacteria inhabiting the animal digestive tracts like *Helicobacter pylori* and *Campylobacter* spp. can develop competence *in vitro*, the biological significance of horizontal acquisition of extracellular DNA in the digestive system remains unclear.

Uptake of DNA by Bacteria Present in Food

Food provides an excellent growth substrate for many types of microorganisms. Some evidence exists for the uptake of extracellular DNA by bacteria residing in food. Natural transformation of *B. subtilis*, a common contaminant in milk, ranges from 10^{-4} to 10^{-3} transformants per recipient, with the highest frequency of 3×10^{-3} obtained in chocolate milk after an incubation time of 12 h at 37 °C. Plasmid transfer by natural transformation of *E. coli* in various foods, including milk, soy drink, tomato juice, carrot juice, vegetable juice, supernatants of canned cabbage, soy beans, shrimps, and various mixtures of canned vegetables, has also been shown to occur. Natural transformation occurred in all growth substrates, although at variable frequencies. Typically, fewer than 10^{-8} transformants per recipient cell were observed, a frequency three or four orders of magnitude lower than those of transformation experiments conducted under optimized laboratory conditions. No correlation was found between the content of divalent ions such as Ca^{2+}, which are considered prerequisites for artificial transformation of *E. coli*, in the foods and transformation frequencies. The above studies exemplify that food sources may provide the conditions required for natural transformation to occur and that the bacterial contaminants of food can experience competence-inducing growth conditions.

Some Limitations of the DNA Uptake Model Systems Used

The data generated from transformation assays used to determine the availability and uptake of DNA in bacterial recipients are linked to the specific laboratory conditions employed. The informative value of such data on the DNA uptake processes occurring under natural conditions must be assessed case by case. Some technical constraints often embedded in the studies include the practice of adding bacterial recipients of a single strain, and often also DNA and nutrients, to a laboratory-maintained microcosm under semiartificial conditions. With few exceptions, studies of DNA uptake by natural transformation have been conducted *in vitro*, or in microcosms intended to represent soil, plants, or the gastrointestinal tract. The ability to establish representative bacterial habitat locations and population sizes after introducing externally cultured bacterial recipients into structurally organized microcosm models containing heterogeneously distributed indigenous bacteria is questionable. Few, if any, studies have examined DNA uptake processes in indigenous microbial communities with the DNA naturally present and spontaneously released on site. Moreover, the concerted action of DNA uptake and selection in determining the genetic compositions of bacterial populations undergoing adaptation to environmental changes is rarely studied in combination.

Several explanations for the dearth of such studies can be found. Most importantly, the range of gene transfer frequencies that can be practically measured in the laboratory is around 1 transformants per 10^8–10^9 exposed cells. The lower relative cell densities of a given species that are usually present in natural bacterial communities effectively limit the gene transfer frequencies that can be measured. Another limitation is that only a fraction of the indigenous bacteria from any given environment can be cultivated for subsequent selection, making DNA uptake difficult to verify in uncultivable bacteria. Moreover, the use of selectable marker genes as a tool for DNA uptake identification is sometimes problematic due to frequently encountered background resistance to most antibiotics and a limited ability of rare transformants to adequately express the marker gene. Only a minor portion of bacteria from any given complex environment is, therefore, susceptible to positive selection by antibiotics and will take part in any screen for competence development. Because most detection methods of DNA transfer in bacteria are based on the uptake of resistance genes, the enumeration procedures usually performed on selective media disturb the sites and conditions that induced the gene transfer. Methods are now being developed for the *in situ* detection of discrete bacterial cells that allow potential DNA uptake events to be identified and quantified *in situ*. For instance, fluorescent protein (e.g., green fluorescent protein) markers have been used to monitor horizontal acquisition of single genes *in situ*.

Most studies on natural transformation have been performed with monocultures with high population densities of the bacterium grown under laboratory conditions. Transforming bacterial species may, however, have specific requirements for competence development and resource utilization in complex environments. Hence, conditions conducive for gene transfer by natural transformation vary and the conditions established in the laboratory may not be those promoting competence development under natural conditions. It, therefore, follows that species currently thought to be nontransformable may show competence under yet unmonitored conditions.

Factors Affecting the Stable Uptake of DNA in Single Bacterial Cells

Molecular barriers define the sexual isolation or the degree to which two bacteria are prevented from exchanging genetic material by recombination. Sexual isolation may be expressed in terms of observed recombination frequencies between two bacteria, or more specifically as the ratio of homogamic recombination within species to heterogamic recombination between species. Molecular barriers act upon chromosomal DNA uptake from its first interaction with the outside of the cell through its integration in a heritable state.

Studies of natural transformation with various degrees of divergent DNA (**Table 1**) show that low DNA sequence similarity is a strong barrier to the integration of chromosomal DNA in bacteria. Indeed, studies have consistently demonstrated an absolute requirement for sequence similarity for detectable transformation of bacteria with chromosomal DNA. For some bacteria, the minimum length of 100% DNA similarity necessary to facilitate efficient resolution of heteroduplex molecules has been experimentally determined and is referred to as the minimum efficient processing segment (MEPS). The minimum amount of base-pairing required for a single

Table 1 Some examples of interspecies/heterogamic recombination in bacteria

Transformation-mediated recombination in chromosomally localized loci	*Sequence divergence of recombining DNA molecules (%)*
Bacillus licheniformis and *B. mojavensis*	17
Streptococcus intermedius and *S. pneumoniae*	18
Acinetobacter sp. strain 01B0 and *Acinetobacter* sp. strain ADP1	20
Rhodococcus erythropolis and *Acinetobacter* sp.	24
Other recombination systems in nonchromosomally localized loci	
In vitro recombination between M13 and fd phage	3
Conjugal gene transfer between *Escherichia coli* and *Salmonella typhimurium*	17
S. pneumoniae and various cloned Streptococcal fragments located on plasmids	18
E. coli and *S. typhimurium* genes located on compatible multicopy plasmids	25
λ phage – plasmid cointegration in *E. coli*	35[a]

[a] 263/405 bp.

region to initate heteroduplex formation with linear DNA substrates is 153 bp in *S. pneumoniae* and 183 bp in *A. baylyi*. Fewer details are available for the exact minimum requirements for double-sided DNA similarity for heteroduplex formation to occur. Such quantification is also not straightforward as it is likely to depend on the species and strain, and the specific nucleotide composition (GC%, etc.) of the recombining regions. For circular DNA intermediates (present in double-stranded form in the cytoplasm), DNA similarities of as little as 25 bp can mediate a single-crossover event and integration into the bacterial chromosome.

Base pairing of the single-stranded donor DNA with the complementary recipient strand within the heteroduplex molecule results in the formation of a double-stranded region that is recognized and acted upon both by host restriction modification (RM). With the exception of *H. influenzae*, transforming DNA becomes single-stranded during the translocation process, which should theoretically render it insensitive to immediate digestion by restriction enzymes. Nevertheless, studies indicate that restriction systems reduce heterogamic transformation sixfold in *B. subtilis* and 100-fold in *P. stutzeri*.

The methyl-directed mismatch repair (MMR) systems of bacteria contribute to sexual isolation by binding to mismatches in heteroduplex regions formed during DNA-strand invasion, resulting in abortion of heterospecific recombination events. While the contribution of MMR to sexual isolation in some bacteria (e.g., *E. coli*) is large, it appears to be less important in naturally transformable bacteria such as *B. subtilis*, *S. pneumoniae*, *P. stutzeri*, and *A. baylyi*. In bacteria, defective MMR can lead to increased rates of heterogamic recombination in addition to increased rates of mutation, as cells are unable to identify/repair mismatches in heteroduplex DNA formed during heterologous strand invasion. For instance, inactivation of the *mutS* gene, encoding the protein that recognizes and binds to mismatches, results in less than 20-fold increase in heterogamic transformation and recombination with DNA substrates of 20% sequence divergence in *A. baylyi*, threefold in *B. subtilis* (at 14.5% sequence divergence), and sixfold in *P. stutzeri* (at 14.6% sequence divergence). These results are in contrast to plasmid–phage recombination systems in *E. coli* or *Salmonella enterica* subspecies *Typhimurium*, where *mutS* deletions increase heterogamic recombination rates by 1000- to 10 000-fold. Reasons for this dichotomy in MMR inhibition of heterogamic recombination are unknown, but it may be due to the molecular machinery of the individual bacterium or due to the nature of DNA interacting with the recipient chromosome (single-stranded in *B. subtilis*, *P. stutzeri*, and *A. baylyi* vs. double-stranded during phage or plasmid integration in *E. coli* and *S. enterica*).

Considerations of the Long-Term Persistence of Horizontally Acquired DNA in Bacterial Populations

The observed frequencies of DNA uptake, and prevalence and diversity of transformants in a bacterial population are determined by (1) the molecular barriers to natural transformation (see above); (2) the limited access to relevant DNA as determined by the nucleotide diversity present and accessible (physical and geographic isolation); and (3) the reduced fitness of bacterial transformants, after uptake of most random DNA fragments, that leads to their removal from the bacterial population by purifying selection.

Experimental transformation studies have most often focused on quantifying the frequencies at which particular gene segments may be taken up by bacteria at one or a few chromosomal loci. The DNA uptake rates quantified are often described in the scientific literature as ‘low’ to ‘high’. It is important to note that such grading only reflects the range of frequencies that can be practically measured in limited sample sizes in the laboratory. The grading is not, as often erroneously implied, linked to the subsequent biological impact of the acquired DNA in the bacterial population. It may be that frequencies below those that can be observed in a 24–48-h DNA uptake experiment in the laboratory are significant in the long run. Most successful horizontal gene transfer (HGT) events identified through comparative DNA analysis of bacterial genomes are estimated to have occurred over a timescale of millions of years. Therefore, experimentally measured DNA uptake frequencies collected over minute timescales in the laboratory may not necessarily provide relevant information to understand the occurrence and biological impact of infrequent DNA uptake events taking place in larger bacterial populations over several years.

In addition to considerations of the relevant timescale of DNA uptake events, the timescale of transformant population expansion may also be considerably longer than what can be practically measured in the laboratory. The long-term fate of any DNA uptake event/transformant is determined by selection and genetic drift and not the DNA uptake frequencies themselves. This is because it is the population trajectories of the descendants of the primary transformants that will determine the long-term survival and impact of the transformant. For instance, single DNA uptake events may take place within the time span of a few bacterial generations. Nevertheless, it may subsequently take many thousands of generations and, hence, many years before the trait will become widespread in the overall bacterial population after clonal division and directional selection of the transformant. The latter process can only be approximated through modeling (e.g., probabilistic or deterministic) of bacterial populations, if the strength of the selection of the transformant is known. The timescale needed to understand DNA uptake processes as they occur naturally depends on the objective of the study, the anticipated directional selection of the transformant bacteria, and their population structure, size, and generation time.

Predictors of DNA Uptake in Bacteria and Implications for the Release of Genetically Modified Microbes

Genomic information and laboratory evidence support both the occurrence of genetic recombination between bacteria of variable genetic relatedness and the existence of mechanistic and selective barriers that maintain divergence between bacterial lineages. A challenge in microbiology is to reconcile these two observations to assess the recombinogenic potential and impact thereof between different bacterial entities. The ability to accurately predict the potential for two separately evolving bacterial lineages to undergo genetic exchange remains, however, in its 'adolescence'. This is illustrated by our inability to predict the impact of future HGT events on bacterial genome evolution, although data on DNA uptake frequencies can be extracted from many experimental model systems. Knowledge of the factors governing horizontal transfer and selection of transformed bacteria is, nevertheless, crucial to the evaluation of the potential for unintended spread of recombinant DNA from genetically modified microbes (GMM). GMM can offer benefits in, for example, improving food production and utilization and in disease prevention. A potential hazard arising from the indiscriminate use of GMM is adverse alteration of indigenous bacterial populations after horizontal transfer of recombinant DNA (recDNA). For instance, the potential of unintended transfer of antibiotic resistance determinants, used as selective markers in the construction of GMMs, to other members of the microbial community has been extensively evaluated.

Most countries and many international organizations have developed legislation and recommendations on the types of assessments necessary prior to the commercial use of GMMs. For instance, the Codex Alimentarius Commission of the United Nations have developed principles for risk analysis and guidelines for safety assessments of foods derived from modern biotechnology, including recDNA microorganisms. In most assessments of genetically modified organisms (GMO), the starting point is the familiarity and the history of safe use/behavior/consumption of the parent organism. The concept of 'substantial equivalence' is used to structure the assessment relative to the conventional counterpart and to focus the assessment on the determination of similarities and differences. Thus, risk assessment is based on the introduced biological changes in the modified organism and does not usually address the biological safety of the parent microorganism itself. In the context of unintended horizontal transfer of recDNA, some general safety recommendations for the construction of GMMs have been made.

1. The genetic modification performed should be limited to the intended trait, and the final GMM or product thereof should not contain unnecessary DNA sequences, for example, antibiotic marker genes, DNA sequences that confer or stimulate genetic mobility of the recDNA, or DNA sequences that can confer or affect pathogenic properties.
2. A chromosomal location of the recDNA is desired because extrachromosomal elements such as plasmids are often self-transferable or mobilizable between cells and species. Plasmids also harbor replication functions that ensure their stability in an extracellular state over bacterial generation.
3. The use of recDNA (genes) that mediate a selective advantage to unintended bacterial recipients should be avoided to prevent dissemination of recDNA in bacterial communities.

The general recommendations made above are precaution-based and seek to minimize both the likelihood of uptake of recDNA in bacterial populations, and the potential positive selection of unintended bacterial transformants carrying recDNA, if rare DNA uptake events occurred. To reduce the likelihood of occurrence of unintended horizontal gene transfer, the recDNA may be inserted into the chromosome in the absence of sequences conferring mobility. Empirical and theoretical data provide a basis for the identification of parameters of importance for limiting the transfer potential of chromosomal DNA between bacteria. Nucleotide differences in DNA sequence between bacterial strains and species are probably the most important barrier to recombination of chromosomal DNA in bacteria. The lack of DNA sequence similarity between the donor and the recipient DNA preclude the ability of the transforming (rec)DNA fragment to form a heteroduplex with the recipient chromosome. In general, experimental studies in transformable bacteria have shown that DNA substrates with more than approximately 30% sequence divergence will not be successfully integrated in the recipient genome. Knowledge of the sequence divergence between species can be a useful predictor of their overall recombination potential. Different approaches are available to estimate the overall DNA sequence similarity between bacteria, including DNA–DNA hybridization, whole genome comparisons, or comparisons of housekeeping genes, including 16S rRNA. While predictors of overall genomic sequence relatedness can be useful to understand the potential for chromosomal recombination between species, knowledge of local sequence divergence and conservation in the area spanning the recDNA will more accurately reflect its recombination potential into unintended recipients.

The technical approaches to limit the potential of unintended recDNA transfer from GMM are required because a precise understanding of bacterial fitness of unintended recipients of recDNA is lacking. As discussed above, it is not the transfer frequencies of chromosomal DNA that will cause a biological impact, but directional positive selection of transformants carrying recDNA. Integrated DNA fragments (including recDNA constructs) are likely to cause phenotypic changes that negatively affect the transformant fitness relative to the larger bacterial population and communities. Transformants carrying recDNA that are not competitive in growth and cell division will not remain in the larger bacterial population for long. Identifying conditions that may promote positive selection of transformants is, therefore, essential to understand the fate of recDNA in any bacterial population. The use of recDNA (genes) that is likely to mediate a selective advantage to unintended bacterial recipients should be avoided to prevent unchecked dissemination of recDNA in bacterial communities. Unfortunately, available methodology does not readily facilitate the collection of empirical data on the selection coefficients of GMMs and unintended bacterial recipients of recDNA in agricultural settings or the gastrointestinal tract. Expert evaluation and inference from a history of safe use and expected behavior (of both the recDNA donor[s] and the recDNA recipient bacterium), therefore, remain an essential component in the case-by-case assessment of GMMs.

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Influenza

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Glossary

aerosol Very fine particles of liquid or solid in suspension.

droplet Tiny drops that sediment quickly.

epidemic Outbreak of a disease affecting many persons at the same time in a locality.

fomite Inanimate object capable to transmit an infectious agent from one person to another.

pandemic An epidemic of global proportions.

Abbreviations

CNS	central nervous system
HA	hemagglutinin
NA	neuraminidase
NEP	nuclear export protein
NP	nucleoprotein
RNP	ribonucleoprotein

Defining Statement

This article summarizes the disease and pathogenesis of influenza virus infections, an important respiratory pathogen that causes yearly epidemics and pandemics every 10–50 years in humans. Animal influenza viruses and their contribution to the generation of pandemic strains of influenza viruses are also discussed.

Introduction

Influenza has been recognized as a human respiratory disease for more than 2000 years, and its symptoms were described by Hippocrates, in the fifth century BC. However, the causative agent of the disease, the influenza virus, was first isolated in swine by Shope in 1930 and shortly after in humans by Smith, Andrewes, and Laidlaw in 1933. Influenza viruses not only affect humans, but they are also known to infect different bird species, pigs, horses, seals, whales, dogs, cats, and other small and large carnivore mammals. Today we know that there are three antigenically distinct types of influenza viruses circulating in humans: influenza A, B, and C viruses. Influenza C viruses are believed to cause mainly asymptomatic or mild respiratory infections in humans. Influenza A and B viruses are responsible for the annual epidemics of influenza in humans, with one type being prevalent at a particular year. Often, the prevalent type is an influenza A virus, but approximately every 4–5 years, influenza B viruses become the predominant circulating influenza virus in humans. A hallmark of influenza viruses is their ability to cause global worldwide epidemics affecting a great number of individuals, or pandemics. Pandemic episodes are solely attributed to influenza A viruses and they occur every 10–60 years. The 1918 influenza pandemic is considered to be the most devastating infectious disease affecting humans in a short period of time. Human pandemics are characterized by the generation of a new strain of human influenza A virus containing antigenic determinants from avian influenza A viruses, for which there is little or no preexisting immunity in humans. In this article we will discuss epidemic human influenza and animal influenza, the origin of pandemic influenza, the determinants of influenza virus virulence and pathogenesis, and the measures for the prevention and treatment of influenza.

Human Influenza

Causative Agent

Human influenza is caused by influenza A and B viruses. Although there is no antibody cross-reactivity between the proteins encoded by these two types of influenza viruses, their replication cycle is very similar. Both are negative-strand RNA virus belonging to the orthomyxovirus group whose genome is composed of eight RNA segments, each segment encoding one or two viral proteins. The virions are enveloped, with two types of viral glycoproteins (spikes) inserted in the envelope: the hemagglutinin (HA) and neuraminidase (NA). The HA recognizes the viral receptor, sialic acid-containing molecules, and therefore is responsible for the attachment of the virus to cells. This results in the internalization of the virus into an endosome, where acidification of the pH causes a conformational change of the HA that triggers fusion of viral envelope with the membrane of the endosome, resulting in the injection of the viral genome into the cytoplasm. The viral genomic RNAs are encapsidated by the viral nucleoprotein (NP), in the form of ribonucleoprotein (RNP). The viral RNPs have also bound the viral RNA-dependent RNA polymerase, a complex of three protein subunits, PB2, PB1, and PA. The RNPs are surrounded by a layer of viral matrix protein (M1), but this layer becomes dissociated (uncoating) from the RNPs by its previous exposure to acidic pH just prior to the fusion event. This exposure is mediated by a small viral ion channel or M2 protein (BM2 in the case of influenza B virus) that like the HA and NA is anchored into the viral envelope. The M2 protein transports protons from the acidified endosome to the interior of the virion, resulting in dissociation of the M1 from the viral RNPs. Uncoated RNPs are transported to the nucleus where replication and transcription takes place through the activity of the viral RNA polymerase, which synthesizes a replicative positive-strand intermediate as well as mRNAs. Among the newly synthesized viral proteins, the viral nuclear export protein, or NEP, is responsible for the exit of the newly synthesized viral RNPs from the nucleus back to the cytoplasm, and budding at the plasma membrane results in the formation of new enveloped virions that spread to new cells. The NA is required for efficient spreading by removing sialic acids present in newly synthesized virions, which otherwise will be bound by HAs from adjacent virions, resulting in viral clumping. Both influenza A and B virus synthesize a viral nonstructural protein or NS1 in infected cells that subverts the cellular antiviral response. In addition, most of the strains of influenza A virus encode a short nonstructural viral polypeptide, PB1-F2, that localizes to the mitochondria and modulates the apoptotic cellular pathways.

Disease

Influenza virus in humans is transmitted through the respiratory route. Most cases of influenza virus infections will result in the development of classical respiratory disease symptoms, starting around 2 days after infection, such as chills, malaise, fatigue, headache, cough, nasal congestion, and sneezing. In many instances, infected individuals are confined to bed for several days, with fever and aches throughout their bodies. Sometimes, these symptoms are accompanied by nausea and vomiting, especially in children. In most of the cases, symptoms subside after 5 days, but severe infections and pneumonia can also occur, specially in the very young, the elderly, and in people with chronic medical conditions, such as asthma, diabetes, or heart disease. Despite available vaccines and antivirals, it is estimated that approximately 50 million people become infected yearly with influenza, with more than 200 000 hospitalizations and about 36 000 deaths just in the United States.

Infection by influenza virus results in pathological changes in the respiratory tract, with severity of the disease mostly being attributed to infection of the lower respiratory tract. These changes are characterized by inflammation of the larynx, trachea, and bronchi due to infiltration of neutrophils and mononuclear cells, accompanied by desquamation of ciliated epithelial cells lining the trachea and bronchi. Cases of primary viral pneumonia are characterized by bronchiolitis, interstitial pneumonitis, and alveolitis, in some instances of a necrotizing nature, with viral antigen being detected in type 1 and 2 pneumocytes and in alveolar macrophages. These pathological changes result in high fever, cyanosis, and hypoxemia and in the most severe cases lead to death.

Combined viral–bacterial pneumonia and secondary bacterial pneumonia are also associated with severe disease. Influenza virus infection appears to predispose the lower respiratory tract to colonization by pathogenic bacteria, most likely by exposing receptors for bacterial attachment. Most of the bacterial complications are caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Hemophilus influenzae*. The fatality rate jumps in these cases to $\sim$10%, reaching almost 50% in the case of coinfections with *S. aureus*. Otitis media, sinusitis, croup, pneumonia, and myositis are frequent complications of influenza virus infection in children. Pregnant women have a predisposition to severe influenza virus infection during the second and third trimester of pregnancy, but viruses do not appear to infect the fetus.

Influenza virus infections in humans are usually confined to the respiratory tract, and most of the extrapulmonary manifestations of influenza in cases of severe disease are believed to be a consequence of respiratory failure. In rare occasions, viruses can be detected at low titers in blood. Viral encephalitis has been described in some instances, especially among influenza virus-infected children in Japan, but most of the central nervous system (CNS) symptoms sometimes seen during influenza virus infections are attributed to metabolic effects from hypoxia and severe pulmonary infections, and not to direct infection of the CNS. Reye syndrome is a poorly understood noninflammatory encephalopathy with high fatality rate associated with the use of aspirin during influenza B virus infections in children.

Seasonal Epidemic Influenza

Yearly epidemics of influenza occur during the winter season, except for tropical and subtropical countries, where influenza virus is isolated sporadically from humans all year long. The reasons for the seasonality of influenza are poorly understood, and may include a combination of different factors, including virus stability in aerosols at colder temperatures, more close contact among people in winter than in summer, and possibly lower levels of host defenses during the winter time. Recently, by using a guinea pig model of airborne transmission of influenza virus, it was shown that lower temperatures and humidity facilitates influenza virus transmission.

Currently, there are three antigenically distinct influenza viruses circulating in humans, influenza B virus and influenza A virus of the H1N1 and H3N2 subtypes (**Figure 1**). The HA, and to some lower extent the NA, is the main target of neutralizing antibodies elicited after influenza virus infection. Different subtypes of influenza A viruses have been described, characterized by HA and NA proteins belonging to specific antigenic groups. A total of 16 subtypes for the HA, H1 to H16, and a total of 9 subtypes for the NA, N1 to N9, have been described. Neutralizing antibodies against one HA or NA subtype do not cross-react with another subtype. The prevalence of influenza B and influenza A H1N1 and H3N2 virus infections varies from year to year, with A/H3N2 viruses in general being more prevalent and also more virulent, followed by B and by A/H1N1 viruses, but this pattern changes in some years. Infection with one of the three types/subtypes of influenza virus does not result in protective immunity against the other types/subtypes, but it has been shown to confer long-lasting protection against the particular infecting strain. In fact, neutralizing antibodies against the H1N1 strain infecting humans in 1918 are still detected almost 100 years later in individuals that were exposed in 1918 to this virus. Nevertheless, influenza A/H1N1, A/H3N2, and B viruses are able to reinfect the same person previously exposed to these viruses. This is possible due to the accumulation of changes from year to year in the antigenic sites of the HA of these viruses that are responsible for partial evasion of preexisting immunity against older strains. This process has been named antigenic drift, and as a result, the circulating influenza virus strains in humans at any given year are antigenically different from those circulating in the previous years. Due to the antigenic drift, the annual influenza virus vaccine, composed of three components (B, A/H3N2, and A/H1N1), requires updation from year to year to mirror the antigenicity of the currently circulating virus strains.

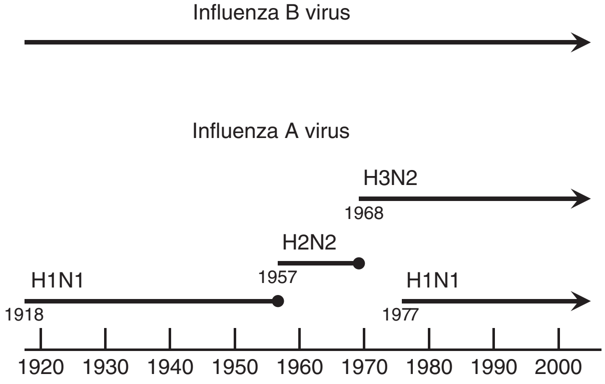

Figure 1 Epidemiology of human influenza virus since the last century.

Pandemic Influenza

Most of the concerns with human influenza virus infections relate to the ability of this virus to cause human pandemics, some of which have been known to be of devastating consequences, such as the 1918 pandemic. There have been three well-documented influenza pandemic episodes during the last 100 years, in 1918, 1957, and 1968 (**Figure 1**). The influenza A viruses causing these pandemics belonged to subtypes H1N1 (1918), H2N2 (1957), and H3N2 (1968). During these pandemic episodes, a new virus subtype started to circulate in humans, and therefore, preexisting immunity against the previously circulating subtype, which antigenically is very different, was of little help to prevent and mitigate infections with the new subtype. With the introduction of a new subtype in humans (or antigenic shift), the previous subtype disappeared from circulation in humans. Pandemic episodes have been characterized by higher number of human infections as well as higher disease severity. In 1977, human H1N1 influenza viruses resumed circulation in humans, and since then they coexist in humans with H3N2 viruses.

While antigenic drift is due at the molecular level by the accumulation and selection of mutations in the antigenic sites of the HA of previously circulating strains, antigenic shift is caused by reassortment. Influenza virus strains from all known 16 HA and 9 NA subtypes are circulating in avian species. While virus strains infecting avian species are not well adapted to infect and transmit in humans (and vice versa), avian and human influenza virus strains coinfecting the same host can readily exchange RNA segments (reassortment) due to the segmented nature of the viral genome. In 1957 a reassortant influenza virus containing the HA, NA, and PB1 genes derived from an avian H2N2 virus, and the rest of the RNA segments from the previously circulating human H1N1 virus, was generated and was able to infect and transmit in humans, initiating the 1957 H2N2 pandemic.

A similar process occurred in 1968 with a reassortant virus containing the HA and PB1 genes from an avian H3 virus and the rest of the RNA segments from previously circulating human H2N2 viruses, resulting in the 1968 H3N2 pandemic. Pigs are believed to have been the intermediate host where these reassortant pandemic viruses were originated, because they are known to be susceptible to infection with both avian and human influenza virus strains. However, other animal host species may also contribute to reassortment and adaptation processes of influenza viruses, resulting in strains with human pandemic potential.

Very little was known about the 1918 H1N1 human influenza virus pandemic due to the lack of virus isolation procedures during the year of the pandemic. This was changed when the genetic material of the virus was sequenced from lung tissue still available from humans who succumbed to this virus. Not only is the genetic information of this pandemic virus now available, but the use of reverse genetics techniques that allow the rescue of infectious influenza viruses from plasmid DNA has also resulted in the reconstruction of the 1918 virus in the laboratory. The characterization of this virus is offering new clues on the determinants responsible for the severe influenza disease experienced by humans in 1918–19, which resulted in approximately 40 million deaths worldwide during the three waves of this pandemic (see below). The origin of this pandemic virus is still unclear, although based on sequence similarities of the 1918 virus with avian influenza viruses, it has been proposed that the virus jumped through an adaptation process in an unknown host from birds to humans in the absence of reassortment processes. However, the lack of sequence information on human influenza virus strains prior to 1918 precludes our ability to firmly conclude whether this was the case.

Molecular Pathogenesis

As with any infectious disease, severe disease caused by influenza virus depends on the genetic composition of the virus, the genetic composition of the host, and the particular immune status of the host at the time of infection. As already discussed, young infants and the elderly are at high risk of severe influenza virus infection, most likely due at least in part to a weak immune system. Nevertheless, it is also possible that an exacerbated immune response might also be responsible for severe cases of influenza virus infection, and, in fact, higher levels of cytokines and immune cell infiltration in lungs is associated with severe influenza virus infection in humans and in animal models. The reconstruction and characterization of the 1918 virus has allowed studies on the reasons responsible for the high virulence of this virus, which not only was responsible for the death of large amounts of people, but which, in contrast with other human influenza viruses, was also particularly more virulent in adult healthy individuals between 15 and 35 years of age. Despite the lack of sequence signatures in its genes known to be associated with high pathogenicity for avian influenza viruses, the 1918 human influenza virus was highly virulent in mice, ferrets, and macaques. In all cases, virulence was associated with high viral titers in the lungs of these animals, although in general viral replication was restricted to the respiratory tract. High viral titers also resulted in a more profound inflammatory response in the lungs, and in elevated proinflammatory cytokines. Neutrophils and macrophages constituted most of the infiltrating immune cells in the lungs of infected animals. However, elimination of neutrophils and of resident alveolar macrophages in mice infected with 1918 influenza virus resulted in more severe disease, indicating that these cells mainly have a protective role. Of interest, elimination of CCL2, a chemokine involved in monocytic/macrophage recruitment from circulation to the lungs, results in decreased lethality of a mouse-adapted influenza virus in mice, implicating these cells as a cause of immunopathology during influenza virus infections.

Recent studies have shown that although the virulence determinants of the 1918 influenza virus are multigenic, most of its pathogenesis and high replicative characteristics is attributed to the HA, NA, and PB1 genes. Of interest, the PB1 gene encodes not only the PB1 protein, a component of the viral RNA polymerase, but also the PB1-F2 protein, absent in contemporary human H1N1 viruses. The presence of a serine at position 66 of the PB1-F2 polypeptide, only found in the PB1-F2 proteins of the 1918 virus and of the H5N1 viruses causing severe disease in humans in 1997, was shown to increase virulence for primary disease as well as for secondary bacterial pneumonia following influenza virus infection in mice. PB1-F2 is a viral pro-apoptotic factor involved in increased apoptosis of immune cells infected with influenza virus, but the role that this particular amino acid has in PB1-F2 function remains to be elucidated.

Infections of mice and macaques with the 1918 virus are associated with profound changes in host gene expression in the lungs of these animals as early as 24 h postinfection. Although it is still too early to understand how these changes contribute to virulence, it is of interest that the NS1 protein of the 1918 virus appears to be a potent inhibitor of the type I interferon-mediated antiviral response, and its C-terminal residue, which lies in a putative protein–protein interacting domain (PDZ ligand domain), has recently been shown to be involved in increased virulence in mice.

Avian Influenza

Low Pathogenic Avian Influenza Viruses

Although influenza A and B viruses are important human pathogens, influenza A virus is mainly an avian virus, with

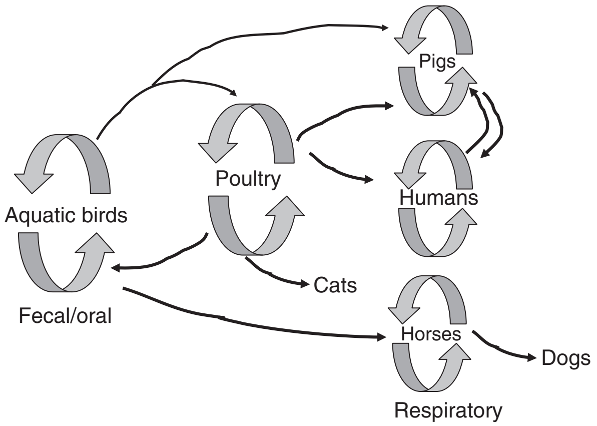

Figure 2 Spread and evolution of influenza A viruses.

all known subtypes of this virus present in wild birds, mainly migratory waterfowl birds. It has been assumed that avian influenza A virus infections in wild birds are asymptomatic, and in contrast to human influenza, where the virus infects the respiratory tract, avian influenza viruses replicate in the intestine tract of birds, and transmit through fecal–oral and fecal–fecal routes of infection. Often, avian influenza viruses from different subtypes establish cycles in domestic avian species (chicken, turkeys, etc.) (**Figure 2**), with different degrees of disease severity, but usually associated with low pathogenicity, with some notable exceptions, described below.

High Pathogenic Avian Influenza Viruses

High pathogenic avian influenza or HPAI is caused only by specific influenza A virus strains belonging to H5 and H7 subtypes. In general, HPAI viruses are not present in wild birds, but when a low pathogenic H5 or H7 strain infects poultry, it can evolve into a high pathogenic strain through the acquisition of a multibasic cleavage site in its HA protein. These viruses cause fulminant disease in chicken and replicate systemically to high titers in multiple organs. The disease in poultry was first described in 1878 in Italy as fowl plague and it was not until 1955 that it was shown to be caused by an influenza A virus.

A multibasic cleavage site in the HA is clearly associated with the high pathogenicity of HPAI virus strains in birds. The viral HA is synthesized as a precursor or HA0, and it requires proteolytic activation into HA1 and HA2 subunits in order to expose the fusion peptide responsible for viral entry. One of the reasons for the tissue restriction of human influenza viruses and of low pathogenic avian influenza viruses to respiratory and intestinal tracts, respectively, is the need for a specific cellular protease able to process the HA0, which is only present in respiratory and intestinal mucosa. By acquiring a multibasic cleavage site, H5 and H7 HAs are now processed by ubiquitous cellular proteases of the furin family, and these viruses become systemic and induce disseminated infections in chicken and other poultry, resulting in high pathogenicity. Although many outbreaks of HPAI have been recorded in poultry in different regions of the world, all of them have been successfully contained with the exception of the HPAI H5N1 outbreak first described in Hong Kong in 1997. Despite the massive culling of poultry in Hong Kong, this virus has continued to circulate among poultry and more recently has been extended to different countries of Southeast Asia, Central Asia, Middle East, Africa, and Europe. HPAI H5N1 viruses are isolated not only from poultry in different areas of the world, but also from dead wild birds, suggesting that wild migratory birds might also play a role in the propagation of H5N1 viruses.

Avian Influenza in Humans

Before 1997 it was assumed that HPAI viruses could not infect and cause severe disease in humans. This concept dramatically changed after the diagnosis of several cases of severe human H5N1 influenza virus infections, many of which were associated with a lethal outcome in Hong Kong. After an initial period of containment, the reemergence of this virus in different areas of the world has been associated with more than 300 cases of severe influenza virus infection in humans, and more than 200 human deaths. Concerns about the human pandemic potential of this virus has prompted global efforts on the development of influenza pandemic preparedness plans, including stockpiling programs of antivirals and H5 vaccines in many countries. Fortunately, despite continuous circulation of H5N1 viruses in poultry, associated with antigenic changes and the appearance of numerous H5N1 clades, only very limited number of infections in humans have been recorded and even less cases of transmission of this virus between humans, only associated with a few infection clusters within members of the same family. Severe infection with H5N1 viruses in humans is likely associated with high levels of exposure to the virus, although it is also possible that predisposing genetic factors in the few individuals who have been infected and developed severe disease exist. Severe infection in humans is associated with high levels of viral replication, viral pneumonia, acute respiratory distress syndrome, and multiorgan dysfunction. Diarrhea episodes are also common during human H5N1 infections, and the virus has been isolated in some cases from feces, indicative of a more extended tissue tropism than regular human influenza virus infections. Viral RNA has also been detected in blood and in CNS in several human cases. However, most of the viral replication in humans appears to be associated with the respiratory tract, and this is also the case in H5N1 experimental inoculations of macaques. In contrast to most avian influenza viruses, H5N1 viruses appear to be highly promiscuous for different mammalian species, and they are known to cause lethal and disseminated disease in mice, cats, ferrets, and tigers.

Nevertheless, HPAI H5N1 viruses are not unique in their ability to cause sporadic severe human disease. In 2003, an outbreak of HPAI H7N7 viruses in chicken in the Netherlands resulted in several human cases of conjunctivitis associated with contact with this virus, and in one human case of fatal severe respiratory infection. Low pathogenic avian H9N2 viruses, widely distributed in poultry in different countries, are also known to infect humans, although not associated with disease. However, any process resulting in selection of viruses of non-H1 and non-H3 subtypes with the ability to infect humans and transmit from human to human will result in a new pandemic, and, therefore, avian influenza viruses need to be closely monitored for any possible changes that might increase their tropism for humans.

While severe infections in birds and mammals with H5N1 HPAI are mainly associated with the multibasic cleavage site of its HA, other viral determinants are known to contribute to increased virulence in mammals. HAs from avian and human influenza viruses recognize sialic acid-containing receptors. However, HAs from human influenza viruses have a preference for binding to sialic acids linked to sugars through alpha2,6 linkages, and HAs from avian influenza viruses have a preference for binding to sialic acids linked to sugars through alpha2,3 linkages. This correlates with the relative abundance of these linkages in the tissues where these viruses replicate: the upper respiratory tract in humans and the intestinal tract in birds. Intriguingly, alpha2,3-linked sialic acids are more abundant in the lower respiratory tract of humans, and this may facilitate replication of H5N1 viruses in the lungs, resulting in severe disease, while restricting replication in the upper respiratory tract, limiting transmission. Also interestingly, changes in receptor specificity of the human 1918 virus from alpha2,6- to alpha2,3-linked sialic acids did not prevent severe disease, but prevented sneezing and aerosol/droplet-mediated transmission in ferrets, suggesting that avian influenza viruses will need to change receptor specificity of their HAs from alpha2,3- to alpha2,6-linked sialic acids as one of the requirements to be transmissible in humans. Polymorphisms in the polymerase genes of HPAI H5 and H7, and especially in the PB2 gene, have also been associated with increased virulence in mammals. A few amino acid changes in the C-terminal PB2 appear to be selected during mammalian or human adaptation of these viruses, resulting in more efficient replication especially at the relative lower temperature of human cells as compared with avian cells. Possible interactions of PB2 with specific host factors have also been postulated and some PB2 adaptive mutations from avian to mammalian hosts have been associated with enhanced recognition of this viral protein by the mammalian nuclear import machinery, required for proper transport of PB2 in mammalian cells. The NS1 gene from H5N1 viruses and from some other avian influenza viruses have also been associated with increased virulence in some mammalian species. The presence of a glutamate at position 92, typical of several H5N1 strains, appears to increase virulence by increasing the ability of the NS1 to mediate resistance to the host interferon-mediated antiviral response. A robust PDZ ligand motif at the C-terminal domain of NS1, typical of most avian influenza viruses, appears to enhance virulence in mice through mechanisms that still are not well understood. Finally, infections of mammals with HPAI H5N1 viruses have been associated with disregulated cytokine responses in macrophages and with lymphocytic depletion, and these factors are also likely to contribute to enhanced disease.

Influenza in Nonhuman Mammalian Species

Besides establishing cycles in human and avian species, influenza A virus strains have successfully established cycles in other mammals (**Figure 2**). The 1918 influenza virus pandemic is believed to have affected pigs in addition to humans, and originated the classical H1N1 swine virus lineage that is still infecting pigs. Swine influenza causes respiratory disease in pigs mainly in combination with other swine respiratory pathogens. Swine influenza is also caused by swine H3N2, H1N2, and modern H1N1 viruses, originated through multiple reassortment processes between human, avian, and classical H1N1 influenza viruses. The recent isolation of H2N3 influenza A viruses in several US pig farms further complicates the epidemiology of swine influenza and raises concerns about the possibility of human infections from pigs with a novel H2 virus, which in theory could initiate a new H2 pandemic.

At least two subtypes of influenza A viruses have been shown to circulate in horses, H7N7 and H3N8, with H3N8 being more prevalent, inducing a respiratory disease in horses similar to the human disease. Equine H3N8 viruses have been associated with an outbreak of influenza in dogs, underscoring the potential of influenza viruses to jump between different species. Other mammals known to be infected by influenza A viruses include ferrets, minks, seals, and whales, and, more recently, cats, tigers, and leopards have been infected with HPAI H5N1 viruses. However, the significance that these animals have in spreading influenza is not clear. Influenza B virus, in contrast to influenza A virus, appears to be specific for humans, with the notable exception of at least one isolation of influenza B viruses from seals.

Treatment and Prevention

Vaccines are available for the prevention of human epidemic influenza virus infection. The vaccine is trivalent and consists of three components: H1N1, H3N2, and influenza B. There are two major types of vaccines in use in the United States, inactivated and live cold-adapted attenuated. The inactivated vaccine is based on egg-grown inactivated virions that are treated for enrichment of HA and NA antigens. It is administered intramuscularly. The cold-adapted vaccine is based on egg-grown live viruses that contain attenuating mutations for growth in the lower respiratory tract by virtue of adaptation to replication at low temperatures, and it is administered intranasally. Both types of vaccines are frequently updated to match the yearly circulating influenza virus strains. Yearly vaccination of high-risk groups is recommended. While both types of vaccines are efficient in inducing protection against seasonal influenza in adults and children, efficacy is decreased in the elderly, one of the groups at risk of severe infections. Recently, more efforts have been dedicated to generate improved influenza virus vaccines, based on the potential advent of a pandemic of unpredicted consequences in humans. The use of reverse genetics techniques for the generation of the master vaccine strains, of cell substrates for the growth of vaccine stocks, of new adjuvants to enhance immunogenicity of inactivated vaccines, of novel recombinant subunit vaccines and novel delivery vectors, of conserved antigenic determinants among different virus strains, and of novel live attenuated virus vaccines are among the different strategies pursued to improve influenza virus vaccine production and/or efficacy.

There are two types of antivirals in use for the treatment of influenza virus infections. The M2 inhibitors, amantadine and rimantadine, block the ion channel activity of this virus protein and prevent viral uncoating. Their use is limited because of widespread resistance against these drugs and side effects. The NA inhibitors, zanamivir and oseltamivir, work by preventing the NA enzymatic activity, resulting in inhibition of viral spread. There are concerns about the possible emergence of resistance against these NA inhibitors, and therefore, novel classes of influenza antivirals based on other viral and maybe even cellular targets are desirable. The therapeutic efficacy of the existing influenza antivirals is limited by the need to treat shortly after infection, after the first onset of symptoms.

Concerns about availability of vaccines and antivirals during a pandemic outbreak of influenza viruses have also prompted to consider nonpharmacological interventions to mitigate the impact of the pandemic. These include quarantine measures, social distancing, and the use of masks and disinfectants, among others. However, more basic knowledge is needed on the relative contribution of fomites versus infectious droplets and infectious aerosols (the latter able to travel distances greater than 3 feet) in the transmission of influenza virus in humans in order to rationally adopt the best possible measures to reduce transmission during a pandemic outbreak.

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Legionella, Bartonella, Haemophilus

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Glossary

alarmone Small guanine nucleotide molecules (i.e., ppGpp and pppGpp) that are produced by bacteria under stress and regulate numerous cellular activities in response. These alarmones are signals for the stringent response that occurs in the presence of amino acid starvation.

atypical pneumonia Infection of the pulmonary airspace that fails to produce the sudden onset and rapid clinical course associated with pneumococcal (''typical'') pneumonia.

BCYE-α Buffered charcoal yeast extract agar supplemented with α-ketoglutarate. This medium is enriched for the isolation of *Legionella* species, and it also contains supplemental iron pyrophosphate and cysteine.

Carrión's disease A geographically isolated disease transmitted by sandflies infected with *Bartonella bacilliformis*, including a severe febrile illness with hemolysis (Oroya fever) and chronic disseminated skin lesions (verruca peruana).

chancroid A sexually transmitted disease caused by *Haemophilus ducreyi* that results in one or more shallow ulcers on the genitals, often internal and asymptomatic in a female reservoir host, but symptomatic and painful on the external genitalia of male contacts.

endocarditis An infection of the internal surface of the heart, usually manifested as a collection of fibrin, platelets, and microorganisms deposited on one of the valve leaflets.

endocytic pathway The process by which substances that are ingested by the cell are directed to their final intracellular destination. In the case of ingested bacteria, this refers to the steps in the delivery of the phagosome to the lysosomal compartment.

epiglottitis A potentially fatal infection of the epiglottis, the flap of tissue that normally covers the opening of the trachea into the pharynx. Swelling of this structure in the presence of bacterial infection can cause asphyxia and death.

hematophagous Literally, 'blood-eating'; referring to the behaviors of some biting arthropods.

hemolytic anemia A loss of erythrocytes in the bloodstream by virtue of a process that causes their rupture or inappropriate removal from the circulation.

mucins Heavily glycosylated large proteins that form massive aggregates to cover mucosal surfaces, providing a barrier to penetration of some microorganisms.

Abbreviations

BA	bacillary angiomatosis
CDC	Center for Disease Control
COPD	chronic obstructive pulmonary disease
CSD	cat scratch disease
ER	endoplasmic reticulum
Hib	*Haemophilus influenzae* type b
IFA	indirect fluorescent antibody
LOS	lipooligosaccharide
NF-κB	nuclear factor-κB
OUP	outer membrane protein
PRP	polyribosylribitol phosphate
TLR	Toll-like receptor

Defining Statement

This article describes three pathogenic Gram-negative bacterial genera, namely *Haemophilus*, a strictly human, mucosal pathogen transmitted by direct contact; *Bartonella*, a zoonotic pathogen transmitted by arthropods that produces variable disease in nonreservoir hosts depending on the competency of the immune system; and *Legionella*, an aquatic, intracellular parasite of free-living protozoa and fortuitous, dead-end pathogen that causes pneumonia in humans with respiratory disease or immunocompromise.

Introduction

This article focuses on three bacterial genera, *Haemophilus*, *Bartonella*, and *Legionella*. These bacteria are all fastidious Gram-negative pathogens, each of which requires specialized conditions for *in vitro* cultivation. However, these organisms share little else in common. As pathogens, they represent a wide range of pathophysiologic and epidemiologic features. For example, *Haemophilus* spp. are primarily extracellular pathogens that infect mucosal surfaces. The species discussed in this article are exclusively human pathogens that are transmitted by respiratory droplets or by direct contact between people. Consequently, they have evolved to adapt to and evade the human immune response. In contrast, *Bartonella* organisms are intracellular pathogens in their natural hosts. They have evolved to be transmitted among the reservoir hosts by arthropod bites and to evade immune clearance in both the reservoir host and the arthropod vector. In contrast, *Bartonella* infections of accidental hosts, such as in humans with cat scratch disease (CSD), are extracellular infections, and the diseases they cause depend on the integrity of the host immune response.

Finally, *Legionella* bacteria are residents of natural biofilms, where they are also occasional intracellular pathogens of free-living amoebae that graze on them. When they are inhaled by humans in the form of aerosols from environmental water sources, they must be able to grow in alveolar macrophages to survive in the lungs. As humans do not transmit the infection to other humans, it is presumed that the bacterial functions that confer the capacity to survive and grow in macrophages evolved in phagocytic protozoa in the natural environment. Hence, these three pathogens represent a strictly human pathogen transmitted by direct contact (*Haemophilus*), a zoonotic pathogen transmitted by arthropods (*Bartonella*), and an environmental bacteria that becomes a fortuitous, dead-end pathogen in humans (*Legionella*). The remainder of this article focuses on the specific factors that mediate the life cycle and pathophysiologic features of these three bacterial species.

Legionella

The Organism

The Legionellaceae are aerobic, Gram-negative bacilli that use amino acids rather than carbohydrates as their preferred energy source. There are nearly 50 species within the genus *Legionella*, the majority of which have been isolated only from environmental, rather than from clinical sources. In the environment, these organisms may inhabit complex communities composed of multiple bacterial species that grow within biofilms. They have been isolated from waters with temperatures ranging from 5 to 50 °C; however, they can grow to abundance at the warmer end of this spectrum, particularly in water distribution systems with water heaters. *Legionella* bacteria are natural parasites of free-living protozoa that dwell in lakes, ponds, or streams and graze on bacteria in biofilms. When *Legionella* organisms are taken up by protozoa, such as the amoeba species *Hartmannella* or *Acanthamoeba*, they are able to evade intracellular degradation and grow within specialized vacuoles in these single-cell eukaryotes. The coexistence of *Legionella* bacteria and a competent amoeba host in the pipes and tanks of potable water systems appears to be a prerequisite for the creation of aerosols, which may result in human infections.

Legionella pneumophila is the most frequently encountered and the best-studied species, as it is associated with most of the human infections, usually in the form of an atypical pneumonia designated Legionnaires' disease (see 'History'). There are 16 distinct serogroups of *L. pneumophila*, with serogroup 1 accounting for the majority of recognized Legionnaires' disease cases. At least 18 other species have caused human infections (e.g., *Legionella micdadei*, *Legionella dumoffi*, and *Legionella bozemanii*), but they are much less prevalent and most often isolated from hospitalized or severely immunocompromised patients. All of the potential pathogens must be able to survive and grow within macrophages in order to cause productive infection in the lungs of mammals, presumably by exploiting pathogenic mechanisms evolved in response to the natural hosts, the protozoa. Dependence on the intracellular niche for growth is a feature shared by the most closely related non-*Legionella* species, *Coxiella burnetii*. *C. burnetii* is an obligate intracellular pathogen that is also a cause of atypical pneumonia.

The Legionellaceae are fastidious in their growth requirements *in vitro*. They are susceptible to noxious substances generated during the autoclaving of agar. Thus, semisolid media are typically prepared with added charcoal to absorb these substances. The standard media are complex, composed of yeast extract, and are buffered to a pH of 6.9 with an organic buffer (ACES) and potassium hydroxide. Supplemental L-cysteine and soluble iron in the form of the pyrophosphate salt are required for growth, and the organisms grow best when α-ketoglutarate is also supplied as a nutritional substrate. Typically, *L. pneumophila* can be isolated on this buffered charcoal yeast extract media (BCYE-α) in 2–5 days. As most normal flora grow abundantly on this rich media, antibiotics may be added to select *Legionella* spp. from nonsterile specimens. Identification of the *L. pneumophila* serogroup or of a non-*L. pneumophila* species is usually made with specific antisera that recognize unique lipopolysaccharide composition.

History

Awareness of the Legionellaceae began after a large outbreak in a downtown Philadelphia hotel that hosted an American Legion Convention in 1976. Over 200 persons were affected, and the case-fatality rate was 15%. The severe respiratory disease affecting the conventioneers thus became known as “Legionnaires’ disease.” However, the etiologic agent, *L. pneumophila* serogroup 1, was not isolated and characterized until the following year at the Center for Disease Control (CDC). The organism was initially passed from postmortem clinical specimens into guinea pigs and subsequently into specialized, enriched media. The association of this organism with disease was suggested by the development of antibodies to this organism in affected patients, detected by using an indirect fluorescent antibody (IFA) test with the fixed bacteria as the capture antigen. The use of the IFA test also permitted CDC investigators to screen stored human sera associated with prior unexplained outbreaks of respiratory infection, several of which were retrospectively diagnosed as Legionnaires’ disease, one of which had occurred at a different convention at the same Philadelphia hotel.

In addition to severe pulmonary disease, outbreaks of self-limited, flu-like illnesses were associated with seroconversion to *L. pneumophila* serogroup 1. This benign response to exposure to *Legionella* organisms has been named ‘Pontiac fever’ (see below for details). Since 1977, the list of *L. pneumophila* serogroups and non-*L. pneumophila* species has gradually lengthened, as new organisms were isolated from patients and from environmental sources.

Epidemiology

With refinements in the media and serology, it was possible to show that Legionnaires’ disease occurs both in discrete outbreaks and in sporadic cases. Water distribution systems were shown to be the predominant environmental sources of human infection (e.g., potable water, water aerosols from showers, sprayers, decorative fountains, and evaporative cooling towers). When outbreaks occur from these sources, exposure of humans is usually very common, but disease among exposed individuals is relatively rare. Accordingly, attack rates in these outbreaks are typically ~5%, suggesting that most humans can clear an inhaled inoculum of *L. pneumophila* without developing disease. In contrast, most individuals who develop Legionnaires’ disease in outbreak situations have one or more underlying conditions that may have disposed them to disease (e.g., advanced age, smoking history, lung disease, heart disease, or immunosuppression).

Pathogenesis – Intracellular Infection

Intracellular infection of free-living amoebae in the natural aquatic environment appears to be a strategy that permits *Legionella* organisms to survive and grow from complex bacterial communities that are frequently grazed upon by the protozoa. Mutants that cannot grow in protozoa have been isolated. Whether these mutants could survive in the aquatic environment is unknown. However, it is clear from laboratory animal experiments that infection in the lungs requires the capacity to infect and to grow in alveolar macrophages. Mutants that cannot grow in cells cannot cause disease. Because *Legionella* bacteria are not transmitted between mammals, it is presumed that the mechanisms that permit growth and development in macrophages evolved first in protozoa.

Transmissive and replicative phases

L. pneumophila alternate between two distinct phases to complete their intracellular life cycle. The regulation of these phases occurs through a complex cascade of gene expression or suppression, which is thoroughly reviewed by Molofsky and Swanson. A key determinant of the phase is the abundance of free amino acids, as amino acids are the primary nutrient source for *L. pneumophila.* When nutrients are abundant, the posttranscriptional regulator CsrA represses traits associated with transmission and cellular invasion (e.g., flagella synthesis and motility, lysosomal evasion, resistance to stress, and cytotoxicity) and promotes bacterial division. In contrast, when nutrients are scarce, the ribosome-associated enzyme RelA is triggered to produce the alarmone (p)ppGpp. This alarmone in turn increases the stationary-phase sigma factor RpoS, and it stimulates a virulence-associated two-component system, LetA/LetS. The latter system induces CsrB, which represses that action of CsrA. Together with the abundance of RpoS, these regulators induce the expression of the transmissive traits mentioned above and inhibit the replication of the bacteria.

As in log-phase growth in nutrient broth, the intracellular niche provides abundant amino acids to the bacteria. Consequently, in both log-phase and intracellular growth, the organisms replicate rapidly, but they do not express flagellar motility or any of the traits required to establish infection in cells. If harvested from these growth states, cells are unable to initiate cell infection *de novo.* As a broth culture approaches stationary phase, or as the intracellular bacteria exhaust the amino acid stores of the cell, (p)ppGpp is produced, the bacteria stop growing, and they express a phenotype that will permit the transmission to a new host cell, that is, motility and cellular invasion traits. Hence, bacteria grown to stationary phase in broth culture or harvested from cells just before lysis are efficient in establishing infection in new host cells.

Intracellular life cycle

Flagellate *L. pneumophila* (in the transmissive phase) are promptly taken up by macrophages on contact (**Figure 1**, step 1). Some strains induce uptake by a novel coiling mechanism in which a pseudopod wraps itself around the bacterium, drawing the organism into the cell while sorting host cell membrane proteins (**Figure 2(a)**). After ingestion, the bacterium escapes from the endocytic pathway, avoiding trafficking to lysosomes. Instead, the *L. pneumophila*-containing vacuole attracts other membranous intracellular organelles (**Figure 1**, steps 2 and 3). Specifically, the cellular proteins, Arf1 and Rab1, which normally attract vesicles from the endoplasmic reticulum (ER) to the *cis*-Golgi, are instead recruited to the membrane of the *L. pneumophila*-containing vacuole. Within a few hours, the vacuolar membrane enlarges by fusion with redirected ER vesicles. In addition, the membrane is studded circumferentially with ribosomes (**Figure 2(b)**). The ER vesicles contain nascent proteins, and it is speculated that the ribosomes may provide polypeptides that the bacteria cleave proteolytically and utilize as nutrients. Amino acids are likely to be abundant at this stage, since the bacteria convert into the nonflagellate, replicative phase and begin to grow and to divide. As replication proceeds and bacterial numbers increase, some fusion with lysosomes may occur; however, late in the course of intracellular replication, there is little deleterious effect of lysosomal contents on bacterial viability. After 24 h, the cell contains large membrane-bound compartments filled with bacteria, many of which have converted again into the transmissive phase (**Figure 1**, step 5; **Figure 2(c)**). Presumably, the resources of the cell are exhausted at this point, causing an increase in the alarmone, (p)ppGpp, which triggers the phase transition. Bacteria in the transmissive phase produce a cytotoxin that induces the release of bacteria from the cell. Finally, the motile progeny are then free to seek out a new host cell (**Figure 1**, step 6).

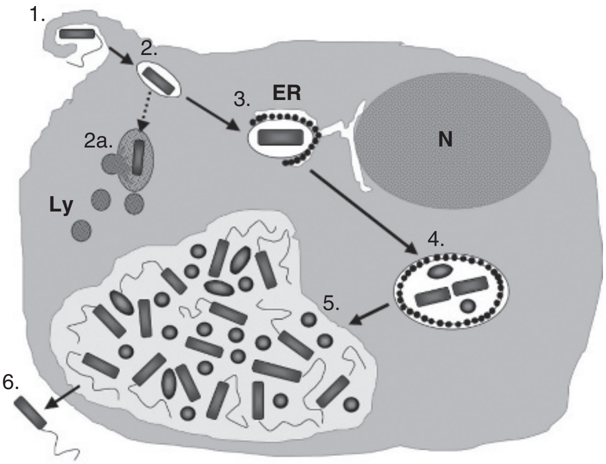

Figure 1 Schematic diagram of events in the *Legionella pneumophila* life cycle in macrophages. Flagellate bacteria in the transmissive phase are taken up by the cell, sometimes by a process of 'coiling' phagocytosis (1). Virulent bacteria within the early phagosome, (2) evade phagolysosomal fusion (2a), and associate with other membranous organelles (3). Endoplasmic reticulum (ER) bearing ribosomes associate with the *L. pneumophila*-containing vacuole, the bacteria are converted into the replicative phase, and intracellular replication begins (3 and 4). At 24 h, large vacuoles displace the cytoplasm of the cell, and the bacteria are converted again into the transmissive phase (5) before they are released from the cell to swim free to the next host cell (6).

Mechanisms of intracellular survival

Survival of *L. pneumophila* in both macrophages and amoebae depends on the integrity of a type IV secretion system, designated as Dot/Icm, and on the effectors that

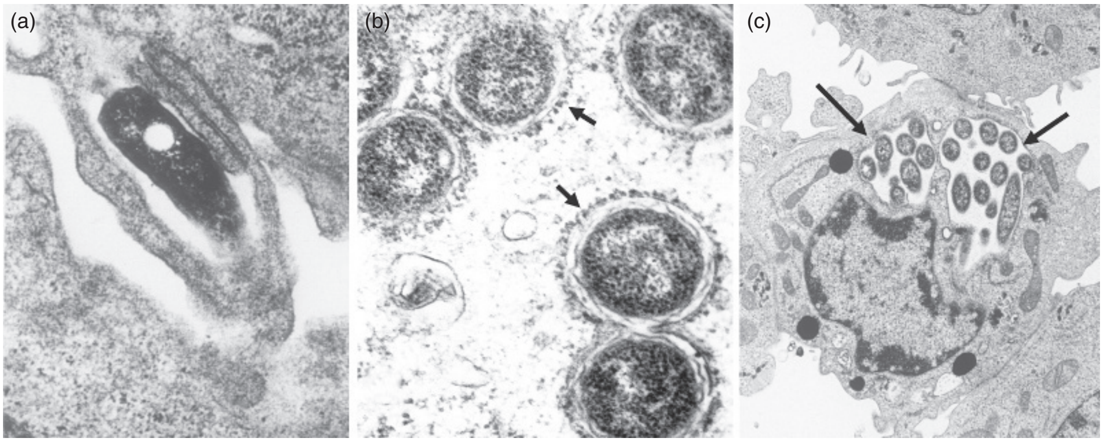

Figure 2 Electron micrographs showing key events in *L. pneumophila* replication in macrophages. (a) An electron-dense bacterium is seen surrounded by a pseudopod coil as it is taken into the cell. (b) Bacteria are seen in individual phagosomes studded with ribosomes (arrows) after endoplasmic reticulum (ER) vesicle recruitment. (c) As intracellular replication proceeds, increasing numbers of bacteria are seen in enlarging vacuoles (arrows).

this system translocates into the host cell cytoplasm. Mutations that abrogate the functions of this secretion system result in the inability of an individual bacterium to escape the endosomal–lysosomal pathway and instead the ingested bacteria are directed to the lysosome within 5 min (**Figures 1** and **2(a)**). More than 30 substrate proteins are now known to be transported by this system, and the functions of some are either known or strongly suspected (**Table 1**). Substrate proteins have been identified using various screens for protein translocation; their functions could sometimes be inferred from their sequence homologies with known effectors. Alternatively, other investigators devised genetic screens to detect specific functions that are thought to mediate the intracellular life cycle events. For example, a genetic screen was used to identify mutants that are unable to recruit Rab1 to the *L. pneumophila*-containing vacuole. Another method identified genes encoding proteins that bind to Rab1. A third approach identified mutations that interfere with membrane traffic to the vacuole in yeast cells. Two Dot/Icm-dependent proteins that redirect secretory vesicles to the *L. pneumophila* vacuole have been identified (RalF and DrrA; **Table 1**). Other Dot/Icm substrates have been shown to interfere with phagosome–lysosome fusion, stimulate host cell antiapoptotic factors to postpone cell death until bacterial replication is exhausted, and facilitate the release of progeny bacteria from the cell (**Table 1**). Many of the substrate proteins translocated by Dot/Icm likely have redundant functions; the functions of several others are currently unknown.

Consequences of Infection

Legionnaires' disease

Infection of humans with *L. pneumophila* is usually acquired by the inhalation of aerosolized bacteria directly into the lower airways. The minimal infectious inoculum and form of the infectious particle (e.g., individual bacteria, amoebae containing bacteria, and isolated amoebic vesicles containing bacteria) are unknown; however, *L. pneumophila* within amoebae are more infectious than broth-grown bacteria alone when equivalent inocula are administered by intratracheal injection in laboratory animals.

As the infection of pulmonary alveolar macrophages with *L. pneumophila* proceeds, inflammatory cells (monocytes and neutrophils) are recruited into the lung, and a fibrinous exudate develops in the airways. Eventually alveoli coalesce into microabscesses. Most of the damage is likely to be a product of the vigorous inflammatory response. The organisms produce a lipopolysaccharide that is mildly endotoxic. They also elaborate a major extracellular protease that is potentially cytotoxic; however, this molecule has not been associated with damage to the lung during infection. The outcome of infection is dependent on the treatment and on the immune status of the individual host.

Host response to infection

The immunocompetent host can mount an innate response to *L. pneumophila* infection of macrophages. In mice, this response is, in part, dependent on the presence of assembled flagella that triggers cytosolic pattern recognition proteins (i.e., naip-5). The activity of the Dot/Icm system also appears to be an early and potent stimulus for apoptosis by the activation of caspase-3. However, *L. pneumophila* also produces antiapoptotic factors that also stimulate cytokine production through the nuclear factor-κB (NF-κB) pathway. Tumor necrosis factor-α produced by this activation may have an autocrine effect on the infected cell that limits infection. Thus, the immunocompetent host may deal with inhaled *L. pneumophila* through purely innate mechanisms. This may explain why patients with Legionnaires' disease tend to be smokers, elderly, and/or immunosuppressed or impaired hosts.

Table 1 Selected substrates of the Dot/Icm type IV secretion system

Function	*Protein*	*Mechanism*
Vacuolar modification	RalF	Exchange factor for Arf GTPase
	DrrA	Exchange factor for Rab1 GTPase
	LidA	Binds to Rab1 GTPase
	SidJ	Facilitates recruitment of ER vesicles
Inhibition of lysosome fusion	VipA	Inhibits lysosomal trafficking (in yeast)
	VipF	Inhibits lysosomal trafficking (in yeast)
	VipD	Inhibits lysosomal trafficking (in yeast)
Prevention of host cell apoptosis	SdhA	Forestalls host cell apoptosis
	SidF	Forestalls host cell apoptosis
Release from the host cell	LepA	Facilitates lytic release of bacteria from protozoa
	LepB	Facilitates lytic release of bacteria from protozoa; inactivates Rab1

Once infection has been established, the growth of *L. pneumophila* within macrophages must be controlled by a Th1-dependent acquired immune response with interferon-γ as the major effector. The restriction of intracellular growth caused by interferon-γ is a result of the sequestration of intracellular iron induced by this cytokine and can be overcome experimentally by providing abundant transferrin-iron. The role of humoral immunity in Legionnaires' disease is secondary. However, specific antibodies may target any extracellular bacteria to neutrophils, where intracellular replication does not occur, rather than mononuclear phagocytes, where infection may flourish.

Pontiac fever

After the discovery of *L. pneumophila* in 1977, an outbreak of illness at the county health department in Pontiac, Michigan, in 1968 was subsequently found to be associated with seroconversion to *L. pneumophila* serogroup 1. The epidemic illness in Pontiac that affected 95% of the building occupants was remarkably different from Legionnaires' disease. It can be noted that the patients reported self-limited, flu-like symptoms that lasted for only 2–5 days. Both healthy and high-risk individuals were exposed and became ill, but none died. In more recent outbreaks of Pontiac fever elsewhere, *Legionella* bacteria cannot be isolated from affected patients, suggesting that illness may be a result of inhaling nonviable organisms. The exact pathogenesis of this illness remains a mystery.

Diagnosis, Treatment, and Prevention

Legionnaires' disease can be diagnosed definitively by the isolation of *Legionella* bacteria from the respiratory tract, although the sensitivity of this approach is poor unless the specimens for culture are collected by bronchoscopy. Moreover, from a practical point of view, culture diagnosis is not helpful clinically, because it is not timely, requiring 3–4 days of incubation before *Legionella* colonies appear on selective media. Currently, infection with *L. pneumophila* serogroup 1 can be diagnosed rapidly and with great sensitivity by a urine antigen test that detects serogroup-specific lipopolysaccharide epitopes. This specific assay is useful in clinical practice because it is rapid and accurate and also because serogroup 1 accounts for more than half of all cases of Legionnaires' disease.

Although *Legionella* organisms are susceptible to most antibiotics *in vitro*, many agents (e.g., all β-lactams and aminoglycosides) are of little or no use *in vivo* because they cannot penetrate into cells where most *Legionella* bacterial replication takes place. Antibiotics that achieve high intracellular levels, such as macrolides, fluoroquinolones, and tetracyclines, are clinically useful. There is currently no vaccine against Legionnaires' disease. However, because many patients who develop Legionnaires' disease are immunocompromised and have inadequate cell-mediated immune responses, the very population that one would wish to protect with a vaccine are also those who would be least likely to profit from it.

Bartonella

The Organism

The *Bartonella* bacteria are facultative Gram-negative bacilli of the class Alphaproteobacteria and are thus most closely related to *Brucella*, *Rhizobium*, and *Agrobacterium* spp. *Bartonella* species have evolved to infect and to establish carriage in specific mammalian host species, although they may also cause disease when transferred to a nonnatural host. In the native host, the anatomical site of persistence of the infection is not known; however, the bacteria may persist in the bloodstream without producing overt sepsis by replicating within erythrocytes. Their presence in the bloodstream (within erythrocytes) enables transmission between natural hosts by arthropod vectors. Several *Bartonella* species have been identified, but only a handful have been observed to infect humans, and only three are known to be important causes of human disease – *Bartonella henselae*, *Bartonella bacilliformis*, and *Bartonella quintana*.

History

In parts of Andean South America, a papular warty skin disorder known as verruca peruana was recognized since pre-Columbian times. An epidemic of a febrile illness associated with hemolytic anemia (known as Oroya fever) occurred among railroad workers in a similar geographic distribution during the late nineteenth century. The common etiology of these two disorders was established in a famous autoexperiment performed by a Peruvian medical student. Daniel Carrión injected himself with lesional material from a patient with verruca peruana; he then developed Oroya fever and died. Subsequently, the biphasic disease caused by this organism has been designated as Carrión's disease in recognition of Daniel Carrión's audacious experiment. In 1909, the erythrocyte-associated bacteria that cause Oroya fever were observed by microbiologist Alberto Barton and later designated as *B. bacilliformis*. *B. bacilliformis* was the only member of the genus *Bartonella* for the next 80 years.

Until 1990, bacillary angiomatosis (BA) was an unexplained opportunistic infection of patients with advanced AIDS. It is a disease that causes persistent fever, malaise, and skin lesions reminiscent of verruca peruana. The focal lesions of BA feature abundant proliferation of small blood vessels, infiltration of inflammatory cells, and abundant silver-staining bacteria. The identity of the bacteria in these lesions was revealed by using a set of universal bacterial primers to amplify 16S rDNA from human tissues affected by BA. The sequence of the amplified DNA was then compared with a databank of known 16S RNA sequences. The closest match was with *Rochalimaea quintana*, the cause of Trench fever, a louse-borne infection encountered primarily during World War I. Consequently, the related agent associated with BA was initially placed in the same genus as *Rochalimaea henselae*. When the close identity of both *R. quintana* and *R. henselae* with *B. bacilliformis* and other zoonotic *Bartonella* species was appreciated, all of these agents were reassigned to the genus *Bartonella* and the genus *Rochalimaea* was eliminated.

Perhaps the most intriguing consequence of the identification and eventual cultivation of *B. henselae* was the discovery that it also caused a more common and benign disease in immunocompetent individuals, that is, CSD. For decades, the etiology of CSD was obscure and widely debated. However, after *B. henselae* was isolated from the tissues of BA patients, it was possible to demonstrate a serologic response to this agent in virtually all patients with CSD. *B. henselae* was also detected in lymph nodes of patients with CSD and in the blood of healthy cats both by cultivation *in vitro* and by PCR. Ironically, the unification of these two disparate pathological entities is like a twentieth century echo of the unification of verruca peruana and Oroya fever, demonstrated tragically by Daniel Carrión's autoexperiment 100 years earlier.

Epidemiology

The occurrence and range of human *Bartonella* infection depend on the geographical location of the natural hosts and the vectors of transmission (**Table 2**). Humans are thought to be the exclusive natural hosts for *B. bacilliformis* and *B. quintana*. *B. bacilliformis* infection is limited geographically to the Western Andean slopes, probably by the distribution of competent sandfly vectors. *B. quintana* is transmitted by the human body louse; the infection is therefore restricted to regions and populations where human infestation with ectoparasites is common, such as among inner-city homeless populations or in refugee situations in Africa. Because *B. henselae* primarily infects domestic cats and is transmitted by cat fleas, the disease is cosmopolitan in distribution, wherever cats are kept as pets. There are numerous other *Bartonella* species that primarily infect other small mammals; however, human contact with these animals and their ectoparasites is uncommon, and human infection is extremely rare.

Pathogenesis

Infection in the natural host

In their natural hosts, most of the *Bartonella* species cause minimal disease manifestations or no disease at all. A notable exception is *B. bacilliformis* infection in humans in which a fatal hemolytic anemia (Oroya fever) can occur. Yet, even with this potentially dangerous infection, there are selected human hosts who become bacteremic, but remain minimally symptomatic. It is not known what factors determine whether a human host will suffer a severe and fatal illness or remain asymptomatic and become part of the reservoir.

The natural host typically becomes infected by the bite of an infected blood-feeding arthropod (**Figure 3**). Adherence of the organisms to both endothelial and epithelial cells is mediated by a *Bartonella* nonfimbrial adhesin, BadA. The same protein also mediates adherence to various extracellular matrix proteins. For most species, the primary site of infection is thought to be the vascular endothelium at the site of inoculation. This assumption is based on the observation that *Bartonella* bacteria readily proliferate in contact with endothelial cells *in vitro*, and they are taken up into a membrane-bound vesicle within these cells. As in the *L. pneumophila* cell interactions described above, *Bartonella*–endothelial–cell interactions are mediated by the activity of a type IV secretion system homologous to the *vir* locus of *Agrobacterium tumefaciens*. This system and at least one set of its translocated effector proteins (BepA-G) are involved in the induction of cytoskeletal rearrangements, establishment of intracellular infection, and suppression of apoptosis. Once established, the primary nidus of infection (presumed to be intraendothelial) serves as a source for successive, synchronized waves of bloodstream infection every few days.

With each synchronized wave of bacteremia, some of the organisms reinfect the other endothelial cells, contributing to the persistence of infection at the primary nidus. More importantly for the transmission of the infection from the

Table 2 Major *Bartonella* species, their hosts, and arthropod vectors

Species	*Natural host*	*Arthropod vector*	*Human disease*
Bartonella bacilliformis	Humans	Sandflies	Oroya fever, verruca peruana
Bartonella quintana	Humans	Lice	Urban trench fever, endocarditis, bacillary angiomatosis (BA)
Bartonella henselae	Cats	Fleas	Cat scratch disease (CSD), bacillary angiomatosis (BA), retinitis
Bartonella vinsonii	Mice, dogs	Ticks	Bacteremia, endocarditis
Bartonella elizabethae	Rats	?	Endocarditis, retinitis

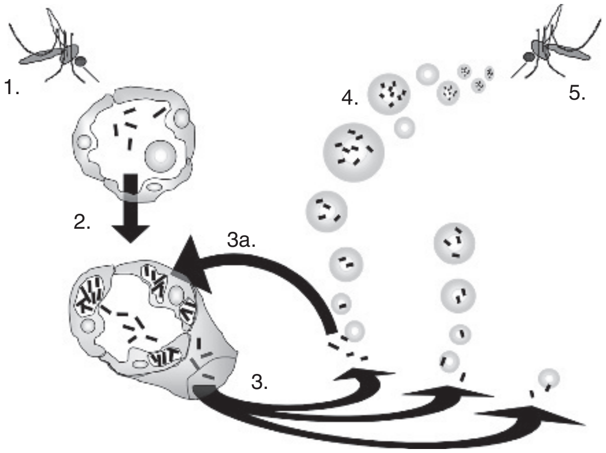

Figure 3 Infection of *Bartonella* in the natural host. The organism is inoculated into the susceptible host by a biting hematophagous arthropod, for example, sandfly, flea, louse, or tick, depending on the *Bartonella* species. (1) Bacteria adhere to and invade endothelial cells at or near the site of inoculation, (2) creating a primary nidus of intracellular growth and source of synchronized release of bacteria into the circulation every few days. Circulating *Bartonella* organisms infect red blood cells (3), and grow to large numbers in the bloodstream (4). Alternatively, some bacteria may reenter endothelial cells to maintain the primary nidus of infection (3a). Senescent, infected red blood cells serve as a source for transfer to an uninfected vector arthropod (5) to complete the cycle.

natural host, many of the bacteria released into the bloodstream invade and grow within erythrocytes. The type IV secretion system mentioned above is also essential for the establishment of erythrocyte infection. The bacteria persist in erythrocytes until these cells become senescent and are removed from circulation. In this way, the bacteria can achieve a high-enough concentration in blood to facilitate efficient uptake when an uninfected hematophagous arthropod feeds on the infected host. As indicated above, bacteremia usually occurs without damaging or septic consequences for the host for at least two reasons. First, the bacteria remain in erythrocytes and are not free to interact with Toll-like receptors (TLR) on circulating leukocytes. Second, the *Bartonella* lipopolysaccharide is modified to be less capable of triggering innate cytokine induction.

The host response to infection partially controls these processes. Antibodies to *Bartonella* cannot clear – and therefore coexist with – intraerythrocytic infection. However, antibodies can prevent successive waves of erythrocytic infection. *Bartonella* also induce the expression of the anti-inflammatory cytokine interleukin-10, which dampens cell-mediated immune responses and facilitates bacterial persistence.

Infection in an accidental host

Erythrocyte infection is not typically a feature of infection in the accidental host. Consequently, the accidental host may have a localized site of symptomatic infection related to the site of entry or a primary infection with septicemia or endocarditis or both. In the case of human *B. henselae* infection, it appears that the immune competency of the host is the most important determinant of disease. Most immunocompetent individuals who are inoculated by a cat scratch develop a minor, transient lesion at the site of the scratch and a highly inflammatory reaction to infection in one or more regional lymph nodes. The immune response is exuberant and limiting. Consequently, the lymph node is infiltrated with acute inflammatory cells, and bacteria are very difficult to find by microscopy. In CSD, the infection may persist for weeks to months, but it is contained at the level of the regional lymph node and is ultimately self-limited.

In contrast to CSD, *B. henselae* infection in an immunocompromised host presents a very different picture. Patients with AIDS cannot contain the infection at the level of the lymph node. Bacteria reach the general circulation, inducing persistent fevers and causing metastatic infection in the skin and liver where the bacteria are abundant in number and easily spotted by microscopy. The same manifestations may occur with infection by *B. quintana* in AIDS patients.

A notable feature of disseminated *Bartonella* infection is vascular proliferation at sites of local bacterial infection. Skin lesions teem with bacteria, and these bacteria have the capacity to induce endothelial cells to proliferate. The result is the development of skin and liver lesions that resemble benign vascular tumors. This process accounts for the warty appearance of verruca peruana (caused by *B. bacilliformis*) and the nearly indistinguishable appearance of *B. henselae* or *B. quintana* skin infection in patients with AIDS. The disease in AIDS is referred to as BA, but the same expression could apply to the Peruvian disease as well.

Diagnosis, Treatment, and Prevention

The *Bartonella* species are not obligate intracellular pathogens and can therefore be isolated on cell-free, artificial media. These organisms will grow slowly on blood agar incubated in 5–10% CO_2. Although culture is feasible, it is insensitive and untimely. The development of colonies may take weeks of incubation. Most cases are therefore diagnosed by serologic testing.

Bartonella bacteria are susceptible to tetracyclines and macrolide antibiotics. Treatment of patients with BA or immunocompromised individuals is essential. However, in most cases of CSD, it is difficult to ascertain whether antimicrobial therapy has any beneficial effect on the duration or outcome of infection. There is no *Bartonella* vaccine for cat owners, but acquisition of the disease can be prevented by the treatment of pet cats and kittens for fleas and by avoidance of contacts that may break the skin or inoculate mucous membranes when handling these pets.

Haemophilus

The Organism

The genus *Haemophilus* includes small, nonmotile, Gram-negative coccobacilli, with *Haemophilus influenzae* being the most medically important member of this genus. As the name *Haemophilus* implies ('blood-loving'), most species require factors present in blood cells for growth *in vitro*. These factors include heme-iron-containing pigments, such as hemin or hematin ('X factor') and NAD or NADP ('Y factor'). Therefore, to isolate *Haemophilus* spp. in the laboratory, clinical samples are inoculated onto chocolate agar, a medium prepared with gently heated blood that has released free X and Y factors from lysed erythrocytes into solution.

Strains of *H. influenzae* may be encapsulated (typeable) or nonencapsulated (nontypeable). There are six known capsular types, type a through type f, but type b is by far the most virulent of the encapsulated strains. The type b capsule is composed of repeating units of polyribosylribitol phosphate (PRP, **Figure 4**). *H. influenzae* type b (Hib) was once the predominant cause of bacterial meningitis in young children but has been largely eliminated by the introduction of a PRP–protein conjugate vaccine in many countries. Strains of nontypeable *H. influenzae* are not uniform and vary in their expression of outer membrane proteins (OMPs). These strains are associated with a high rate of human colonization and in many cases of otitis, sinusitis, and exacerbations of chronic bronchitis. Their prevalence has been unaffected by the use of PRP-conjugate vaccine. Finally, a specific biogroup of nontypeable *H. influenzae*, that is, *aegyptius*, has been associated with purulent conjunctivitis and with a bacteremic condition known as Brazilian purpuric fever.

The genus also includes species that are associated with rare cases of bacteremia, endocarditis, and genital tract infection in humans. These include *Haemophilus parainfluenzae*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, *Haemophilus haemolyticus*, and *Haemophilus parahaemolyticus*. A separate species, *H. ducreyi*, is the cause of the sexually transmitted ulcerative disease, chancroid.

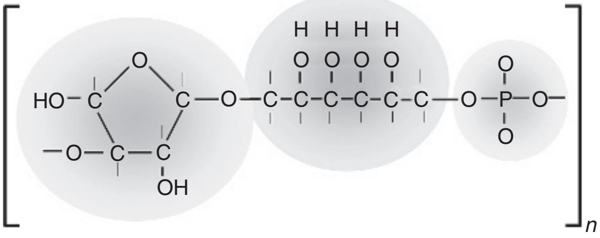

Figure 4 The repeating structure of polyribosylribitol phosphate (PRP) capsule of *H. influenzae*.

History

H. influenzae (previously known as *Bacillus influenzae* or 'Pfeiffer's bacillus') received its species designation because it was isolated from the sputum of patients with influenza. It was even proposed, and briefly accepted, as the cause of the 1918 'Spanish flu' epidemic. Doubt about this association arose after experiments of Peter Olitsky and Frederick Gates of The Rockefeller Institute were published in 1921. These investigators showed that lung disease could be passed from human respiratory secretions to rabbits even after filtration that excluded bacteria. In 1924, Oswald Avery and J. M. Neill (also of the Rockefeller Institute) developed specialized media for growing *B. influenzae* – chocolate agar as described above – and determined the optimal conditions for its cultivation. This contribution allowed for more accurate study of the occurrence of *Haemophilus* infection and led to the realization that *H. influenzae* was a formidable secondary infection in some cases of influenza, but not the primary cause. Obviously, the nomenclature has not changed to reflect this realization.

Epidemiology

H. influenzae is a human pathogen and is distributed worldwide. As noted above, Hib was the predominant cause of bacterial meningitis in young children until the 1990s. Thereafter, the use of capsule-conjugate vaccines virtually eliminated this type of meningitis in countries where it was introduced as part of the universal immunization program for infants (**Figure 5**). The vaccine also reduced the Hib carriage rate dramatically, so much so that there is little Hib circulating in the community. Recent cases of Hib disease in older children who had been previously vaccinated suggest that the lack of a booster effect from natural reexposure to the bacteria circulating in the community may account for the few failures – perhaps a case of the vaccine program being too successful!

Adult infections with *H. influenzae* (e.g., community-acquired pneumonia, otitis, and sinusitis) are associated with nonencapsulated (untypeable) strains. Similarly, respiratory *H. influenzae* infections in populations of immunized children are also usually associated with nontypeable isolates. Fortunately, meningitis is extremely rare with these strains.

Pathogenesis

H. influenzae is transmitted among humans by respiratory droplet spread. The first essential event in a new host is adherence to the upper respiratory epithelium. *H. influenzae* is well equipped for this task; it expresses pili (fimbriae) that bind to fibronectin-coating epithelial

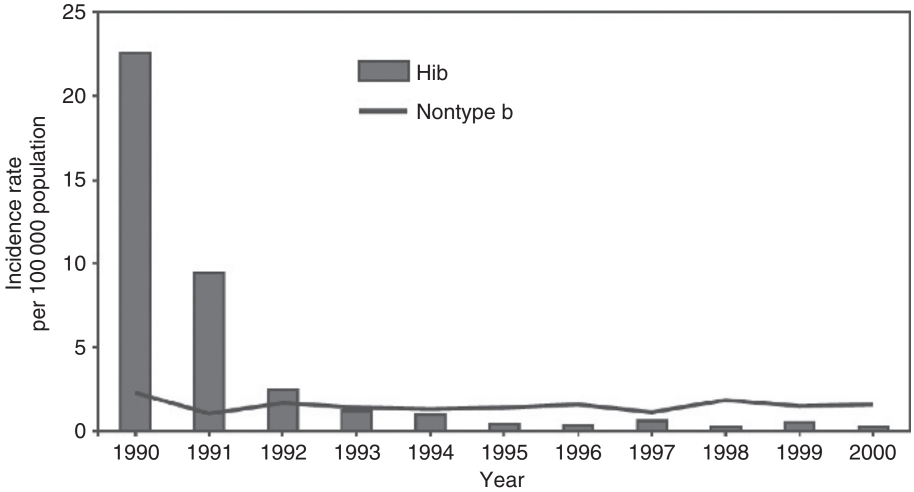

Figure 5 Annual rate of invasive *Haemophilus influenzae* disease (cases per 100 000 population) for the decade following the introduction of type b capsular-conjugate vaccine. Courtesy of CDC, Atlanta, GA.

cells and heparin-binding extracellular matrix proteins. Several nonpilus adhesins have also been identified and bind to alternate receptors such as vitronectin, laminin, or type IV collagen. In addition, several OMPs interact with TLRs (TLR2 and TLR4). A phosphorylcholine residue (ChoP) expressed on the lipooligosaccharide (LOS) interacts with the platelet-activating factor receptor on the host cell surface (**Table 3**). These secondary ligands are also thought to contribute to host cell adherence.

Once attached to the mucosa, the organisms must fend off both innate mechanisms of clearance and acquired mucosal immunity. To address the latter concern, *H. influenzae* elaborates an IgA1 protease that dampens the effects of mucosal antibodies by cleaving IgA at its hinge region. In addition, certain bacterial surface structures mediate the uptake of *H. influenzae* by epithelial cells, providing protection from mucosal immune mechanisms and a route of invasion to the submucosa. The details of the complex host cell signaling during the process of bacterial uptake are beyond the scope of this article. However, it appears that the binding of *H. influenzae* ChoP to the platelet-activating factor receptor is both an important stimulus for pinocytotic uptake of the bacteria and a suppressor of the proinflammatory effects induced by the engagement of OMP-2/OMP-6 and LOS with TLR2 and TLR4, respectively.

Table 3 Factors involved in the pathogenesis of infection by *H. influenzae*

Bacterial factor	*Localization*	*Putative role in pathogenesis*
IgA1 protease	Secreted	Cleaves IgA1 at the hinge region
Haemocin	Secreted	Bacteriocin produced only by Hib that may allow Hib to outcompete nonencapsulated strains at the mucosal surface
Capsule	Surface	Confers serum resistance, antiphagocytic
Hif fimbriae (pili)	Surface organelle	Adherence via cell-associated fibronectin and heparin-binding extracellular matrix proteins
High-molecular-weight adhesins	Surface	HMW1 binds to sialyl-α2,3 hexose; homologous to *Bordetella pertussis* filamentous hemagglutinin
Hap, Hia, and Hsf fibrils	Surface	Adherence to host cells via various receptors
ChoP	LOS	Engages the platelet-activating factor receptor to induce pinocytotic uptake and downregulate Toll-like receptors (anti-inflammatory effect)
Sialyltransferases, SiaB	LOS	Sialylation of LOS confers increased serum resistance
LgtC	LOS	Galactosyltransferase confers increased serum resistance
OMP-2	OM	Engages TLR2
OMP-6	OM	Engages TLR2; target for bactericidal antibodies
OMP-5	OM	Inhibits sloughing of colonized epithelial cells
Major heat shock Protein	Periplasm, OM, etc.	Induces an inflammatory response via TLR2 and MyD88 (NF-κB is translocated to the nucleus)

H. influenzae have been found to have type IV pili that may confer twitching motility and the ability to conjugally transfer genes from a genomic island to a recipient bacterium. Although this conjugative system constitutes a type IV secretion system that transfers DNA from one bacterium to another, there is currently no evidence of translocation of bacterial proteins into host cells to modulate intracellular infection as was described for the *Legionella* spp. and the *Bartonella* spp. in earlier sections of this article.

Some *H. influenzae* surface structures serve as natural targets for innate immune mechanisms. For example, gel-forming mucins on the mucosal surface can bind to and clear piliated *H. influenzae*. To counteract this clearance mechanism, the pilus genes may undergo genetic phase variation, allowing for a subpopulation that is not piliated, and therefore is not susceptible to mucin clearance. The structure of the LOS is also phase variable in a manner that affects survival and virulence. For example, serum resistance is a property that is altered by genetic phase variation of the carbohydrate structure of LOS in nonencapsulated strains of *H. influenzae*. A prominent example of this variation is observed in the galactosyltransferase gene (*lgtC*) that confers serum resistance to the bacterium when it is expressed. This gene is translated into a functional protein only when the number of tetranucleotide repeats in a variable region of the gene conserves an appropriate reading frame. As in other species with similar mechanisms, slipped-strand mispairing provides the genetic mechanism for variation in the number of repeats. Similarly, the addition of sialic acid residues to LOS by sialyltransferases *in vivo* also confers serum resistance and is also subject to phase variation. The phase-variable expression of ChoP on LOS has an opposite effect; the presence of the phosphorylcholine residues on LOS permits the binding of C-reactive proteins and the triggering of the complement system, thus increasing serum sensitivity.

For the encapsulated strains of *H. influenzae*, the key virulence factor that allows for invasive disease and meningitis is the capsule. Nonencapsulated strains tend to cause only localized infections associated with epithelial surfaces and do not have the capacity in normal hosts to metastasize in the bloodstream to internal, sterile sites. There is some evidence of biofilm formation during persistent infection with nonencapsulated strains, but a biofilm-specific exopolysaccharide has not yet been identified.

Pathological Consequences of Infection

Type b, encapsulated strains of *H. influenzae* may cause meningitis, pneumonia, epiglottitis, bacteremia, and septic arthritis in susceptible hosts. However, even in the prevaccine era, these invasive infections were seen almost exclusively in young children. Because of the high frequency of colonization with Hib before extensive vaccine use, most children possessed anticapsular antibodies by the age of 4 years, with or without clinical manifestations of prior infection. Thus, children older than 4 years of age rarely ever developed invasive Hib disease, even in the prevaccine era.

Nontypeable strains of *H. influenzae* cause acute bacterial otitis media, sinusitis, and conjunctivitis in healthy hosts. In addition, these strains may cause opportunistic infections in impaired hosts. For example, they are a common cause of exacerbations of chronic bronchitis in patients with chronic obstructive pulmonary disease (COPD), pneumonia in COPD patients and children in developing countries, neonatal and maternal sepsis, and bacteremia in the elderly or otherwise compromised host.

In all types of *H. influenzae* infection, acute inflammatory responses are the rule. Bacterial factors that trigger these responses include the LOS, which has some endotoxin activity, OMPs that engage TLR2, and the heat shock protein, Hsp70, that also engages TLR2. All of these factors signal the cell to enhance the transfer of NF-κB to the nucleus and to increase the expression of proinflammatory cytokines, provoking a localized acute inflammatory response.

Diagnosis, Treatment, and Prevention

H. influenzae is a relatively fastidious bacterium, but diagnosis typically depends on culture. In addition to their failure to grow on blood agar or MacConkey media (which lack factors X and Y), these bacteria do not survive well outside of the human host. Cultures in hospital settings are frequently negative even when Gram stains are highly suggestive of infection because the bacteria may not survive the prolonged transportation time to the laboratory.

Most *H. influenzae* are susceptible to a variety of antimicrobials, including β-lactams. Ampicillin or amoxicillin is the drug of choice for these strains. However, approximately 17% of strains worldwide are resistant to ampicillin and other penicillins, with frequencies of ampicillin resistance varying considerably in different locales. In the vast majority of these strains, ampicillin resistance is conferred by the production of a β-lactamase enzyme. A very small percentage of strains (<1%) are resistant to ampicillin by virtue of an alteration in a penicillin-binding protein. Ampicillin-resistant strains are susceptible to β-lactam–β-lactamase-inhibitor combinations, second- and third-generation cephalosporins, fluoroquinolones, and some macrolides, any of which can be used successfully to treat *H. influenzae* infections.

As mentioned, vaccination with type b capsular–protein conjugate vaccines has virtually eliminated disease and reduced the carriage of type b strains wherever it has been initiated in infant vaccination programs. There is no current vaccine to protect against infection with nontypeable strains.

Conclusion

The three fastidious Gram-negative pathogens described in this article share elements of their basic structure and the potential to cause disease in humans. They are otherwise distinct in their selection of their preferred hosts and their strategies for survival. Like many bacterial pathogens, these three species cause particularly serious disease in impaired or immunocompromised hosts in whom the pathology may differ dramatically from that in healthy exposed individuals. Although a vaccine has produced a dramatic reduction in the frequency of *H. influenzae* type b infection, preventive strategies for the remaining *H. influenzae* strains and two other species depend on public health and personal lifestyles adjustments.

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Relevant Website

http://www.cdc.gov – Center for Disease Control and Prevention (CDC)

Lipopolysaccharides (Endotoxins)

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Defining Statement
Lipopolysaccharide Structure
Biosynthesis and Assembly of Lipopolysaccharides
Functions and Biological Activities of Lipopolysaccharides
Further Reading

Glossary

core oligosaccharide (core OS) A branched and often phosphorylated oligosaccharide with varying glycan composition that is linked to lipid A. The inner core is more conserved and generally contains 3-deoxy-D-*manno*-octulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep) residues. The outer core OS is more variable in structure.

endotoxin In Gram-negative sepsis, the lipid A components of LPS may stimulate macrophages and endothelial cells to overproduce cytokines and proinflammatory mediators. This can lead to septic shock, a syndrome involving hypotension, coagulopathy, and organ failure.

lipid A An acylated and phosphorylated di- or monosaccharide that forms the hydrophobic part of LPS.

lipooligosaccharide (LOS) A form of LPS often produced by mucosal pathogens, including members of the genera *Neisseria*, *Haemophilus*, *Bordetella*, and others. LOS lacks O-polysaccharide (O-PS) but has oligosaccharide chains extending from the inner core OS. In many bacteria, expression of these chains (and the resulting antigenic epitopes) is phase-variable.

lipopolysaccharide (LPS) An amphiphilic glycolipid found exclusively in Gram-negative bacteria. LPS forms the outer leaflet of the outer membrane in the majority of Gram-negative bacteria.

O-polysaccharide (O-PS) A glycan chain attached to the core OS. Structures of O-PSs vary considerably and give rise to O-antigens that define O-serospecificity in serological typing.

R-LPS Rough LPS, a form of LPS that is truncated by the absence of O-PS and, in some cases, by part of the core OS.

S-LPS Smooth LPS, a form of LPS common in the families Enterobacteriaceae, Pseudomonadaceae, and Vibrionaceae among others. S-LPS has a tripartite structure composed of lipid A, core OS, and O-PS.

Abbreviations

ABC	ATP-binding cassette
Acyl-ACP	Acyl-acyl carrier protein
Ara4N	4-aminoarabinose
BPI	bactericidal/permeability-inducing protein
CAMP	cationic antimicrobial peptide
GalA	galacturonic acid
GlcN	glucosamine
Hep	L-*glycero*-D-*manno*-heptose
IRF	interferon regulatory factor
Kdo	3-deoxy-D-*manno*-octulosonic acid
L-Ara4N	4-amino-4-deoxy-L-arabinose
LBP	LPS-binding protein
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAC	membrane attack complex
MAL	MyD88 adaptor-like protein
mCD14	membrane CD14
MD-2	myeloid differentiation-2
MPL	monophosphoryl lipid
MyD88	myeloid differentiation factor 88
PEtN	phosphoethanolamine
R-LPS	rough LPS
sCD14	soluble form of CD14
S-LPS	smooth LPS
TIR	Toll/interleukin I receptor
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFNβ
UDP-GlcNAc	UDP-*N*-acetylglucosamine
und-P	undecaprenyl phosphate
und-PP	undecaprenyl pyrophosphoryl

Defining Statement

A characteristic feature of Gram-negative bacteria is a complex cell envelope containing an outer membrane. The outer membrane is an asymmetric lipid bilayer that has an inner leaflet containing glycerophospholipids and an outer leaflet whose major component is lipopolysaccharide (LPS), an amphiphilic glycolipid that is unique to Gram-negative bacteria.

Lipopolysaccharide Structure

Lipopolysaccharide (LPS) is often called 'endotoxin', a term first introduced in the nineteenth century to describe the component of Gram-negative bacteria responsible for the pathophysiological phenomena associated with Gram-negative bloodstream infections. Early structural analyses of LPS were driven, in part, by the desire to resolve the identity of the molecule responsible for the endotoxic effect. One of the key breakthroughs in early LPS research came from the establishment and refinement of techniques for the extraction and isolation of LPS by O. Westphal, O. Lüderitz, and F. Bister in the late 1940s and early 1950s. Although other methods have followed, their hot phenol–water extraction method is still commonly used today. This pioneering early work led to the understanding that LPS is responsible for the endotoxic phenomenon, and, equally important, the finding that LPS molecules with similar composition are present in different Gram-negative bacteria. More recent application of high-resolution analytical techniques, such as nuclear magnetic resonance spectroscopy and mass spectrometry, has led to detailed refined structures for LPS molecules from diverse bacteria. It is now clear that there are general structural principles that are highly conserved in the LPSs from different sources, but there is substantial variation when examining the fine structural details.

Extensive research has been performed on the structure, genetics, and biosynthesis of LPS molecules of *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, and these LPSs form a basis for comparative analysis of other LPSs. The LPS of *E. coli* and other members of the family Enterobacteriaceae is organized into three distinct structural domains: O-antigen, core, and lipid A (**Figure 1**). Most lipid A forms have a multiply-acylated diglucosamine backbone that serves as the hydrophobic anchor of LPS. Extending outward from lipid A is the branched and often phosphorylated oligosaccharide known as the core oligosaccharide (core OS). The O-antigen side chain polysaccharide (O-antigen; O-PS) is a polymer of defined repeat units attached to the core OS. The O-PS extends from the surface to form a protective layer. This complete tripartite LPS structure is known as 'smooth LPS' (S-LPS), taking its name after the 'smooth' or shiny colony morphology displayed by enteric bacteria that have S-LPS on their cell surface. Mutants with defects in O-PS or core OS assembly produce truncated LPS molecules but their growth *in vitro* is unaffected. For example, the widely used *E. coli* K-12 strains carry a defect in O-PS biosynthesis. The resulting colonies lack the smooth character, and, therefore, the truncated LPS are widely known as 'rough LPS' (R-LPS) (**Figure 1**). Preparations of LPS from bacteria that produce S-LPS contain a heterogeneous mixture of molecules with differing O-PS chain length and always have a variable amount of truncated R-LPS. This molecular heterogeneity is clearly evident when LPS preparations are examined by SDS-PAGE (**Figure 2**). Some bacteria, particularly mucosal pathogens, naturally lack O-PS chains in their LPS. Their LPS contains oligosaccharide extensions attached to various points of a typical inner core OS, in a form of LPS known as lipooligosaccharides (LOSs) (**Figure 1**).

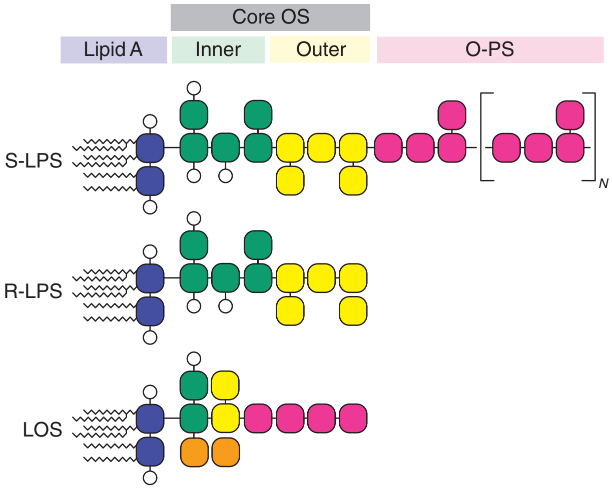


Figure 1 Schematic diagram showing three different forms of LPS molecules. The tripartite S-LPS structure is typical of LPSs produced by members of the Enterobacteriaceae, Pseudomonadaceae, and Vibrionaceae. These bacteria also produce a variable amount of R-LPS that lacks O-PS and, in some cases, part of the core OS. Mucosal pathogens such as *Neisseria* and *Haemophilus* spp. lack O-PS but instead may have phase-variable oligosaccharide extensions attached to the core OS, to form LOS.

The LPS molecules from different bacteria typically show closer structural relationships in the cell-proximal lipid A and inner core OS regions and increasing diversity in the distal outer core OS and O-PS domains. The inner portion of the LPS molecule plays important roles in establishing the essential barrier function of the outer membrane, potentially placing constraints on the extent of chemical, structural variation seen in LPS molecules. The outer part of the LPS molecules interacts with environmental factors, such as the host immune response.

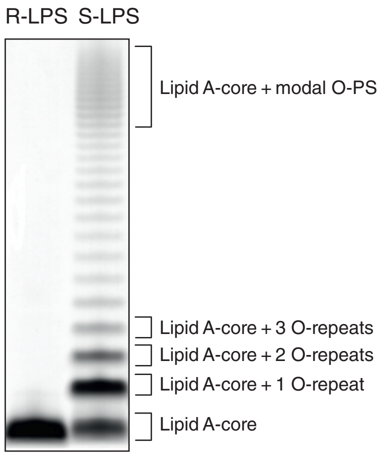

R-LPS
S-LPS
Lipid A-core + modal O-PS
Lipid A-core + 3 O-repeats
Lipid A-core + 2 O-repeats
Lipid A-core + 1 O-repeat
Lipid A-core

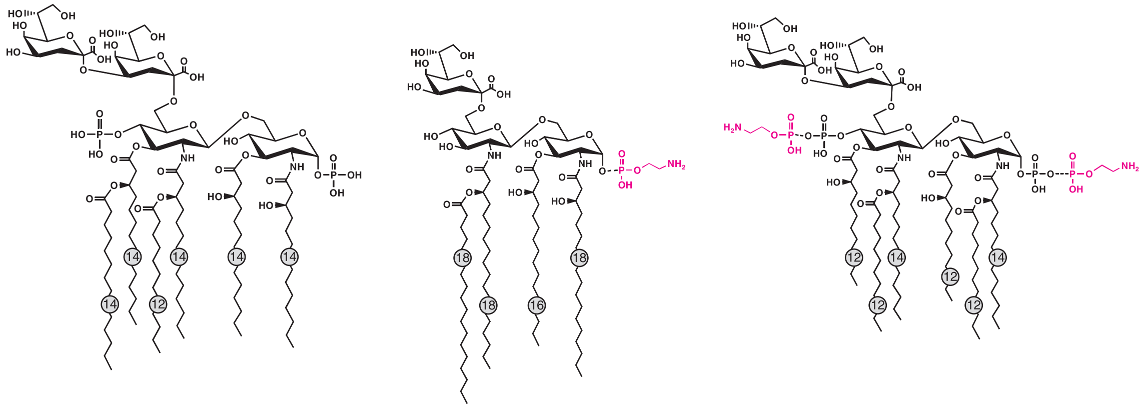
Figure 2 Characteristic SDS-polyacrylamide gel profiles of R-LPS and S-LPS preparations.

These selective pressures may have played a significant role in the diversification of outer LPS structures.

Some bacteria, including *Sphingomonas paucimobilis* and a few examples of treponemes (e.g., *Treponema maritime* and *Borrelia burgdorferi*), have outer membranes that lack LPS molecules entirely. In some cases, key genes for lipid A synthesis are absent from genome sequences and the genetic data are therefore consistent with compositional data. In the case of *S. paucimobilis*, glycosphingolipids probably serve to replace lipid A, and the same may be true for the other examples. In those organisms that have a traditional LPS, it is generally thought that lipid A is essential for viability. However, the universality of this assumption is challenged by the relatively recent identification of a viable *Neisseria meningitidis* mutant containing a defect in an essential step in the lipid A biosynthesis pathway.

Lipid A

During the early stages of endotoxin research, the terms 'lipid A' and 'lipid B' were assigned to components later identified as the lipid moiety of LPS and phosphatidylethanolamine, respectively. Phosphatidylethanolamine often contaminates LPS preparations. The term 'free' lipid A was used to describe the lipid product released by mild-acid hydrolysis of LPS; this procedure selectively cleaves the labile ketosidic linkage between the core OS and lipid A. Until recently, it was the generally held view that free lipid A does not exist on the surfaces of bacteria, but *Francisella tularensis* now provides an exception; only small quantities of the total cellular lipid A are found in a complete LPS structure in this bacterium. Determining the structure of lipid A is difficult due to its microheterogenity as well as its amphipathic properties. However, the lipid A structures from a variety of Gram-negative bacterial species have now been resolved, revealing a family of structurally related glycolipids based on common architectural principles. In enteric bacteria, the backbone of lipid A is formed by a disaccharide comprised of two glucosamine (GlcN) residues joined by a β-(1,6) linkage. The disaccharide backbone is phosphorylated at positions 1 and 4′ in the classic *E. coli* (**Figure 3**) and *Salmonella* LPS structures. These phosphates can be further modified by addition of phospho-ethanolamine (PEtN) or 4-aminoarabinose (Ara4N) in a series of regulated processes (see 'Lipid A modification systems'). The disaccharide backbone is acylated with ester and amide-linked 3-hydroxyl saturated fatty acids (3-OH-$C_{14:0}$). In *E. coli* and *Salmonella*, the fatty acyl chains on the nonreducing GlcN residue are substituted by nonhydroxylated fatty acids, creating an asymmetric arrangement. However, the precise acylation pattern is species-specific (**Figure 3**). The variations in lipid A structure can have an important effect on the



Escherichia coli (unmodified) *Helicobacter pylori* *Neisseria meningitidis*

Figure 3 Structural diversity of representative lipid A molecules found in pathogenic bacteria. The major lipid A species from *Helicobacter pylori* and *Neisseria meningitidis* are compared to that of the unmodified *Escherichia coli* K-12 (i.e., the structure lacking the regulated covalent modifications; **Figure 9**). Partial covalent modifications are indicated with dashed bonds and the enclosed circles indicate the length of each fatty acyl chain.

biology of the organism. Within a given species structural variants arise through complex environmental regulation; the additional glycoforms are not essential for viability in the laboratory but they play important roles in host–bacterium interactions (see ‘O-polysaccharides as a protective barrier’).

In a few organisms, the canonical lipid A format varies. In some representatives of the genera *Rhizobium*, *Brucella*, and *Legionella*, the backbone is comprised of a β-(1,6)-linked disaccharide of 2,3,diamino-2,3-deoxyglucose and long acyl chains (e.g., 27-hydroxyoctacosanoic acid) are found. Other *Rhizobium* species contain a lipid A where the proximal GlcN is oxidized to 2-aminogluconate.

Core Oligosaccharides

For the purpose of discussion of structure–function relationships, the core OS is often divided into inner and outer core regions (**Figures 1** and **4**). The inner core OS of most known LPSs is composed of characteristic residues of 3-deoxy-D-*manno*-octulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (Hep). The first Kdo residue marking the core OS is attached to the 6′-position in lipid A (**Figure 3**). In some nonenteric bacteria, the Kdo residue proximal to lipid A is phosphorylated, or is replaced with the derivative D-*glycero*-D-*talo*-octulosonic acid. These differences may influence the lability of the linkage that is usually cleaved by mild-acid hydrolysis to release lipid A from the intact LPS molecule for structural studies. While Kdo is generally considered to be a diagnostic marker of the inner core, Kdo is also present in the outer core OS of *Klebsiella pneumoniae* LPS (**Figure 4**) and the susceptibility of its linkage to common treatments used to prepare carbohydrate backbones can influence structural determinations. In the Enterobacteriaceae, the base inner core region is highly conserved and the phosphorylated glycan backbone is nonstoichiometrically modified by PEtN and Kdo residues (**Figure 4**). In contrast, the outer core shows more diversity; for example, there are five outer core OS structures in *E. coli* reflecting variations in glycose components and linkages. In most cases, the core OS carries a net negative charge that is

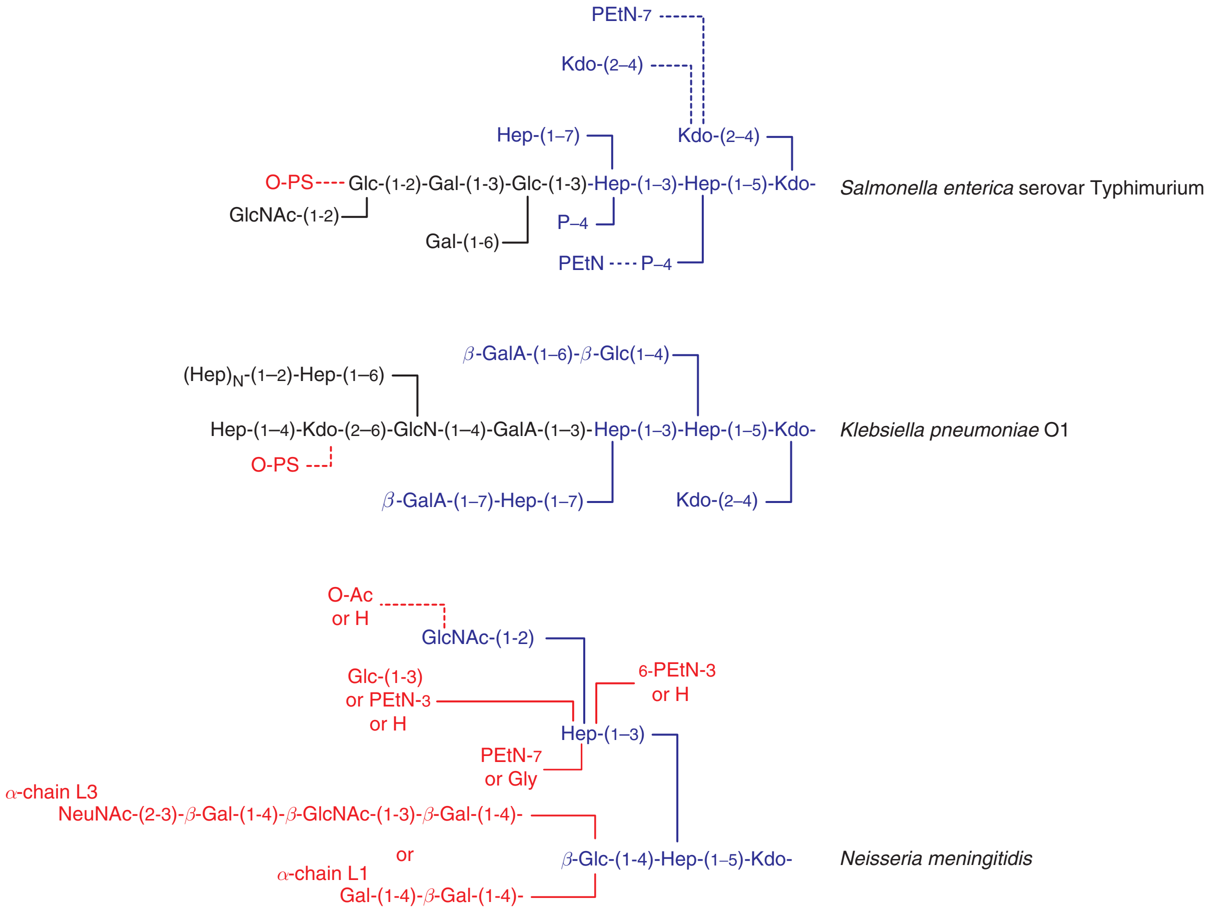

PEtN-7
Kdo-(2–4)
Hep-(1–7)
Kdo-(2–4)
O-PS
Glc-(1-2)-Gal-(1-3)-Glc-(1-3)-Hep-(1–3)-Hep-(1–5)-Kdo-
GlcNAc-(1-2)
Gal-(1-6)
P–4
PEtN
P–4
Salmonella enterica serovar Typhimurium
β-GalA-(1–6)-β-Glc(1–4)
(Hep)N-(1–2)-Hep-(1–6)
Hep-(1–4)-Kdo-(2–6)-GlcN-(1–4)-GalA-(1–3)-Hep-(1–3)-Hep-(1–5)-Kdo-
O-PS
β-GalA-(1–7)-Hep-(1–7)
Kdo-(2–4)
Klebsiella pneumoniae O1
O-Ac
or H
GlcNAc-(1-2)
Glc-(1-3)
or PEtN-3
or H
6-PEtN-3
or H
Hep-(1–3)
PEtN-7
or Gly
α-chain L3
NeuNAc-(2-3)-β-Gal-(1-4)-β-GlcNAc-(1-3)-β-Gal-(1-4)-
or
α-chain L1
Gal-(1-4)-β-Gal-(1-4)-
β-Glc-(1-4)-Hep-(1–5)-Kdo-
Neisseria meningitidis

Figure 4 Structural relationships in the core oligosaccharides from *Salmonella enterica* serovar Typhimurium and *Klebsiella pneumoniae* and the lipooligosaccharide (LOS) of *Neisseria meningitidis*. Inner core residues are shown in blue, outer core (where relevant) in black, and serospecific domains/structures are in red.

important for its function in outer membrane stability (see 'Role of LPS in the outer membrane integrity' and 'O-polysaccharides as a protective barrier'). The charge is contributed by the carboxyl groups of Kdo and phosphate in *E. coli* and *Salmonella*, but *K. pneumoniae* provides one example of a core OS devoid of phosphate. In this bacterium, galacturonic acid (GalA) residues play a critical role in establishing the permeability barrier.

O-Polysaccharides

The O-PS is the most variable portion of the LPS molecule. A remarkable array of novel structures arises from alterations in constituent sugars, linkages, and both complete and partial substitutions with nonsugar residues. The simplest O-PSs contain a disaccharide repeat unit with a single monosaccharide constituent and a single glycosidic linkage type. Complex homopolysaccharides can result from larger repeat units defined by a specific sequence of glycosidic linkages. At their most complex, O-PSs can be heteropolysaccharides in which the repeat units contain several component sugars, together with nonsugar substituents, such as O-acetyl groups and amino acids. The remarkable structural diversity in O-PSs has been exploited in serological classification of isolates from a given bacterial species. In *E. coli*, there are approximately 170 distinct O-serogroups. Although the LPSs from most bacterial strains tend to have a single O-PS type in the same clone, there are a growing number of bacteria being identified for which the lipid A-core serves as an acceptor for two or more different polymeric structures that are coexpressed. The length of the O-PS attached to lipid A-core is heterogeneous but the distribution of chain lengths is both strain- and growth condition-dependent. This is best reflected in the patterns of LPS molecules revealed by SDS-PAGE analysis (**Figure 2**).

Lipooligosaccharides

The LPS of mucosal pathogens such as *Neisseria* and *Haemophilus* spp. is smaller than S-LPS and takes a form that is known as LOS (**Figure 3**). These bacteria generally do not produce S-LPS. As an example, the LOS structure from *N. meningitidis* is given in **Figure 4**. The oligosaccharide chains linked to the inner core OS provide distinct antigenic epitopes giving rise to seven (L1–L7) immunotypes. A common feature in these bacteria is the phenomenon of phase variation, where the LOS epitopes are differentially expressed. The complex glycoforms impact host–pathogen interactions but can give rise to difficulty in arriving at precise LOS structures, unless phase-locked variants are available. Some bacteria (e.g., *Bordetella* spp.) can form both S-LPS and LOS.

Biosynthesis and Assembly of Lipopolysaccharides

As might be anticipated from the structure of LPS, the processes involved in LPS biosynthesis are complex (**Figure 5**). A further complication arises from the fact that most LPS precursors are found in the cytoplasm and the completed molecule must be assembled in a process that traverses the inner and outer membranes, the periplasm and the peptidoglycan layer. In overview, lipid A-core is synthesized in consecutive steps and is then exported across the inner membrane by MsbA, an ATP-binding cassette (ABC) transporter. O-PS is synthesized by one of three pathways (although one has, so far, just a single example). In each, the O-PS is assembled on the lipid acceptor, undecaprenyl phosphate (und-P), and becomes available at the periplasmic face of the inner membrane in the undecaprenyl pyrophosphoryl (und-PP)-linked form. The LPS molecule is completed by transfer of the O-PS to the lipid A-core.

It is generally held that translocation of R-LPS and S-LPS from the periplasm to the cell surface occurs through the same pathway, although the details are scant. Recent studies have begun to shed light on the essential components. These include a conserved outer membrane protein known as LptD (formerly known as Imp or OstA); defects in this protein cause altered envelope permeability properties. Conditional *imp (LptD)* mutants produce membranes with an altered lipid-to-protein ratio, demonstrating a role of LptD in cell envelope biogenesis. LptD exists in a complex with LptE (formerly RlpB), an outer membrane lipoprotein, whose absence gives a phenotype resembling an LptD defect. Several additional proteins are also implicated in LPS translocation; a periplasmic protein (LptA), a cytoplasmic ABC protein (LptB), and three inner membrane proteins LptC (formerly YrbK), LptF (formerly YjpG), and LptG (formerly YjgQ). Both LptF and LptG have been proposed as transmembrane domain proteins that participate with LptB in an ABC protein complex to extract LPS from the inner membrane en route to the outer membrane. LptC is a bitopic inner membrane protein that has also been suggested to play a role in LPS extraction from the inner membrane. LptA has been shown to be an LPS-binding protein (LBP), perhaps serving as a chaperone to mask the hydrophobic domains of lipid A in the aqueous periplasm. However, the mechanisms of action of the other proteins are currently unknown. Two potential models can be envisaged for translocation. In one, the essential machinery comes together to form a complex that spans the envelope and provides specific assembly sites with a direct connection between the inner and outer membranes. This would resemble the proposed 'Bayer junctions' from Manfred Bayer's work in the late 1960s.

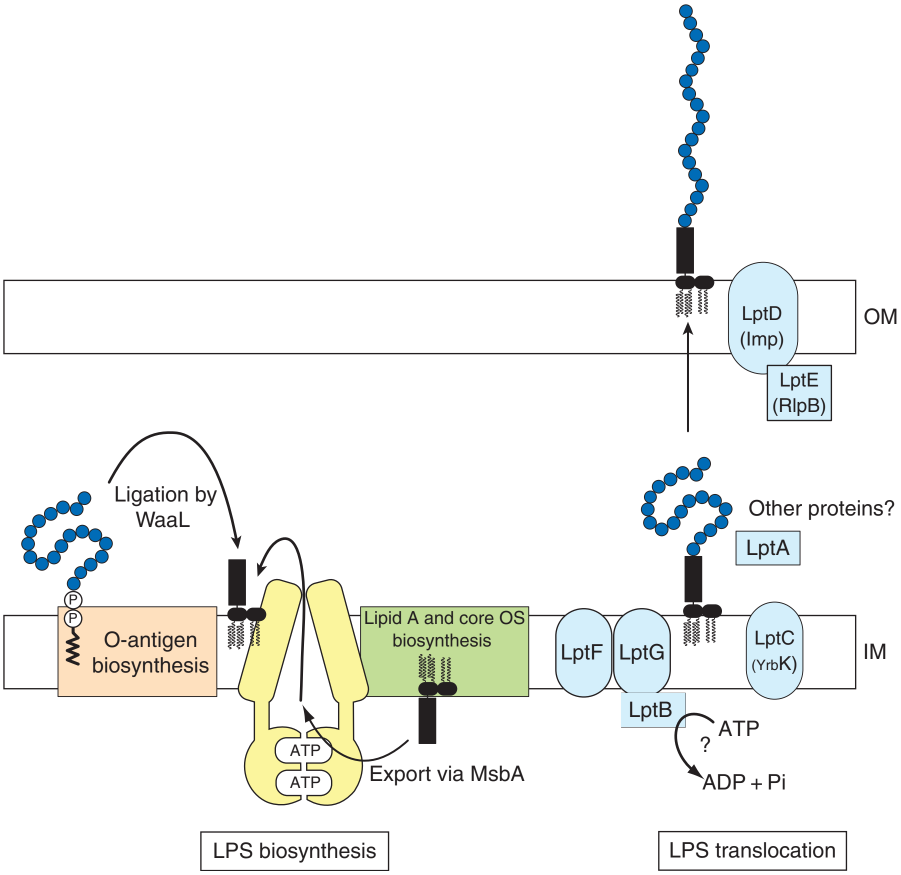


Figure 5 Overview of LPS biosynthesis. The lipid A-core domain is synthesized at the cytoplasmic face of the inner membrane and is exported to the periplasm by MsbA, an ABC transporter. The O-polysaccharide is assembled separately on a lipid carrier (undecaprenyl phosphate) and is presented for ligation to lipid A-core by WaaL in the periplasm. The completed S-LPS molecules and R-LPS are thought to be transported to the cell surface by the same pathway, involving several essential proteins whose mechanism of action is generally unresolved. Nomenclature in parentheses refers to historical gene/protein names; the Lpt designations have been assigned recently and are now in common usage in the field.

Alternatively, the nascent LPS might be translocated via a soluble carrier system similar to the process mediated by the Lol system in assembly of outer membrane lipoproteins. After export across the inner membrane, newly synthesized outer membrane-directed lipoproteins on the periplasmic leaflet of the inner membrane are recognized by the ABC transporter LolCDE complex and released from the inner membrane in the presence of a periplasmic carrier protein, LolA. LolA forms a soluble complex with one molecule of lipoprotein and delivers its cargo to a lipoprotein-specific outer membrane receptor, LolB. LolB receives the lipoprotein from LolA and facilitates its incorporation into the outer membrane. The identification of the potential LPS chaperone, LptA, and the shared relationships in other components suggest obvious similarities between the Lol and LPS translocation systems. This merits further investigation.

Beyond the base (constitutive) assembly system, it is now clear that a variety of important LPS processing and modification reactions occur in the periplasm and outer membrane during the late assembly steps or following insertion into the outer membrane. These have a significant effect on LPS biological activity and cell envelope properties (see ‘Lipid A modification systems’).

Biosynthesis of Lipid A-Core OS

The LPS molecules in most Gram-negative bacteria contain at least one Kdo residue attached to lipid A. Kdo is an essential and highly conserved component found in nearly all LPS structures investigated to date. In *E. coli* and *Salmonella*, the minimal LPS structure required for growth has long been recognized as two Kdo residues attached to lipid A (Kdo_2-lipid A or Re-LPS). Although this has complicated attempts to study the biosynthesis of lipid A, most steps in the pathway are now established through systematic efforts in the laboratory of Chris Raetz; the pathway is widely referred to as the ‘Raetz pathway’. Elucidation of the reaction sequence was made possible by the initial discovery of a novel membrane-associated glycolipid in *E. coli*, 2,3-diacylglucosamine 1-phosphate (also known as lipid X), more than 20 years ago. Lipid X is a diagnostic intermediate that provided essential clues to the biosynthesis strategy. The nine Lpx enzymes of the constitutive Raetz pathway are highly conserved and a single copy of each *lpx* gene can be found in the genome of most Gram-negative bacteria and, interestingly, in plants.

The biosynthetic pathway of *E. coli* Kdo$_2$-lipid A is shown in **Figure 6**. The pathway begins in the cytoplasm of the cell with the acylation of the sugar nucleotide UDP-*N*-acetylglucosamine (UDP-GlcNAc), catalyzed by LpxA. UDP-GlcNAc is also a precursor for peptidoglycan biosynthesis. LpxA and the subsequent acyltransferases use only acyl-acyl carrier protein (acyl-ACP) as the obligatory acyl donor. The second step of the pathway and the first committed step of Kdo$_2$-lipid A biosynthesis is catalyzed by LpxC, a highly conserved deacetylase. LpxC removes the acetyl group from position 2 of UDP-GlcNAc to allow the addition of a second fatty acyl chain catalyzed by LpxD, forming UDP-2,3-diacylglucosamine. LpxH then cleaves the pyrophosphate bond of the UDP-2,3-diacylglucosamine intermediate to yield UMP and lipid X. The disaccharide synthase, LpxB, forms the characteristic β-(1,6) glycosidic linkage by condensing one molecule of lipid X with one molecule of UDP-2,3-diacylglucosamine. Formation of the disaccharide backbone is followed by phosphorylation at position 4′ by a specific kinase, LpxK, resulting in the formation of lipid IV$_A$, a key lipid A precursor. This kinase reaction has played a central role in endotoxin research, in that it allows for the preparation of radioactive ^{32}P-labeled precursors for biochemical studies. A distinctive feature of the Gram-negative bacterial LPS is the presence of the

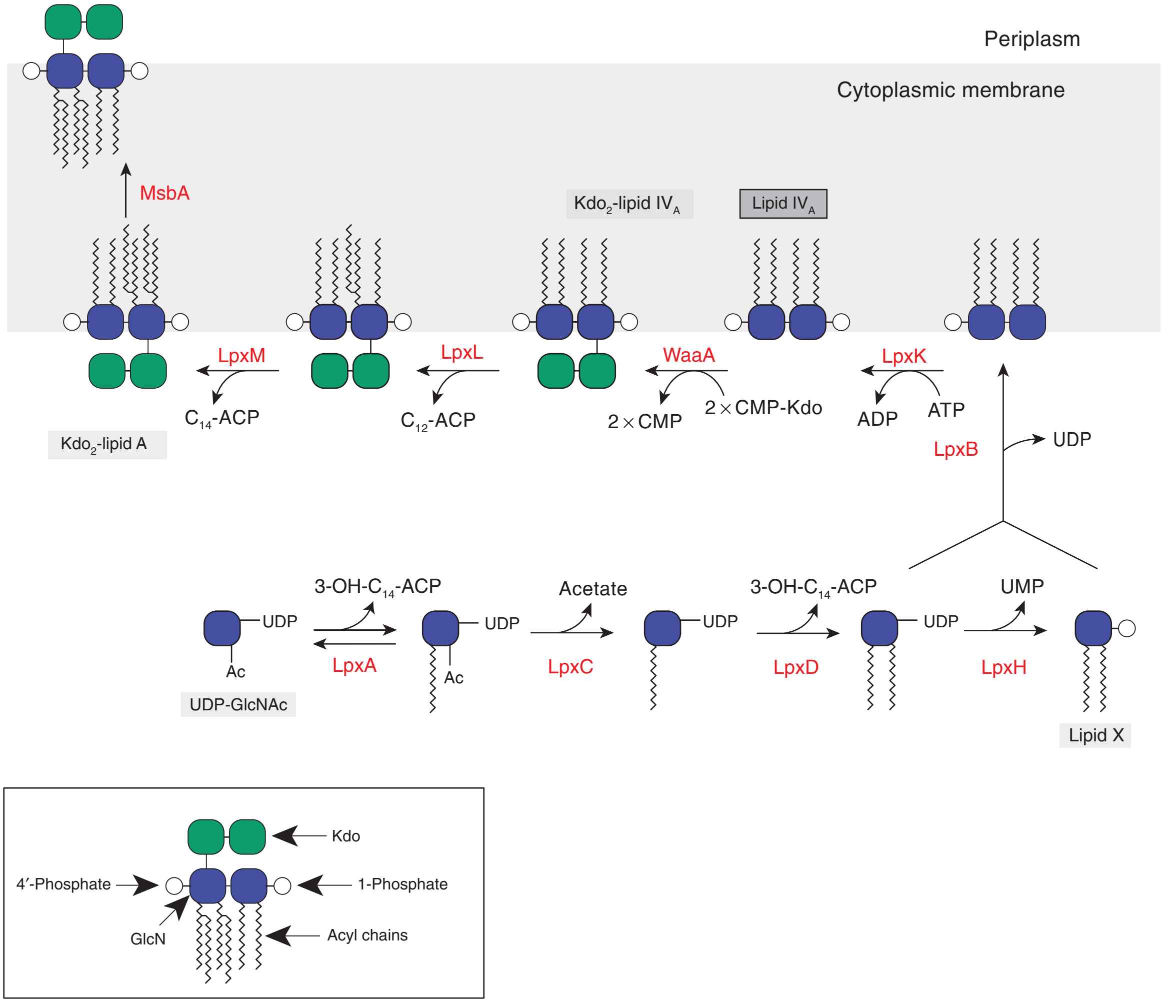

Periplasm
Cytoplasmic membrane
MsbA
Kdo$_2$-lipid IV$_A$
Lipid IV$_A$
LpxM
C$_{14}$-ACP
Kdo$_2$-lipid A
LpxL
C$_{12}$-ACP
WaaA
2 × CMP
2 × CMP-Kdo
LpxK
ADP
ATP
LpxB
UDP
3-OH-C$_{14}$-ACP
UDP
Ac
UDP-GlcNAc
LpxA
UDP
Ac
Acetate
LpxC
UDP
3-OH-C$_{14}$-ACP
LpxD
UDP
UMP
LpxH
Lipid X
Kdo
4′-Phosphate
1-Phosphate
GlcN
Acyl chains

Figure 6 The pathway for biosynthesis of the Kdo$_2$ lipid A domain of LPS in *Escherichia coli* K-12. The enzymes (red) responsible for each step in the pathway are indicated. All have been identified by genetic and biochemical approaches. The completed Kdo$_2$-lipid A provides an acceptor for sequential assembly of the core oligosaccharide at the cytoplasmic face of the inner membrane. Acyl-acyl carrier proteins (acyl-ACPs) serve as the obligate donors for acylation events in the pathway. The ABC-transporter MsbA is responsible for exporting the complete LPS molecule across the inner membrane.

Kdo sugars located at position 6′ of the disaccharide backbone. In *E. coli* and *Salmonella*, transfer of the Kdo sugars is catalyzed by the bifunctional Kdo transferase, WaaA. The Kdo transferase from other organisms, such as *Haemophilus* spp. and *Vibrio* spp., transfer a single Kdo sugar to the disaccharide backbone, whereas in *Chlamydia* spp., WaaA can be trifunctional or even tetrafunctional. All use a CMP-Kdo donor and the structural features that dictate their mono- or multifunctional behaviors are unknown. In *E. coli* and *Salmonella*, the synthesis of Kdo$_2$-lipid A is completed by the addition of two fatty acyl chains by the so-called 'late' acyltransferases, LpxL (lauroyl, C12) and LpxM (myristoyl, C14).

A significant breakthrough in understanding the LPS assembly process and the minimal essential LPS structure in *E. coli* was achieved by the discovery of MsbA, the ABC transporter required for export of lipid A derivatives to the periplasm. MsbA is highly conserved among Gram-negative bacteria and shares homology with the multidrug resistance proteins of eukaryotes. MsbA was first identified by its ability to complement the growth defect of an *lxpL* mutant at elevated temperatures. MsbA is essential for viability in *E. coli*, and inactivation of the transporter in temperature-sensitive mutants results in the accumulation of Kdo$_2$-lipid A and glycerophospholipids in the inner membrane. Some *N. meningitidis* isolates can survive without LPS and therefore tolerate an *msbA* mutation; this bacterium represents an excellent system for studying trafficking of lipid A. Unfortunately, attempts to demonstrate MsbA's lipid A flippase activity in phospholipid vesicles have not yet been successful and remain a major challenge in LPS research. Crystal structures of MsbA transporters from several species are available but the precise structure–function relationships pertaining to lipid A export have not been resolved. A key question remains – how does MsbA handle an amphipathic export substrate; that is, what is the transport solution for a molecule with a highly hydrophobic acylated domain and a polar carbohydrate backbone? MsbA is highly selective for the hexa-acylated LPS forms, but full acylation occurs only after the addition of two Kdo residues to a tetra-acyl lipid A. Thus defects in Kdo addition are manifested as blocks in LPS export. However, it has now been demonstrated that overexpression of MsbA can suppress the lethality of a Kdo-deficient LPS mutant, allowing insertion of tetra-acylated lipid A precursor lipid IV$_A$ into the outer membrane. Presumably the low efficiency of MsbA in exporting the lipid A intermediate is overcome when the ABC transporter is present in high concentrations, by simple mass action. This new discovery redefines the minimal LPS structure required for *E. coli* growth.

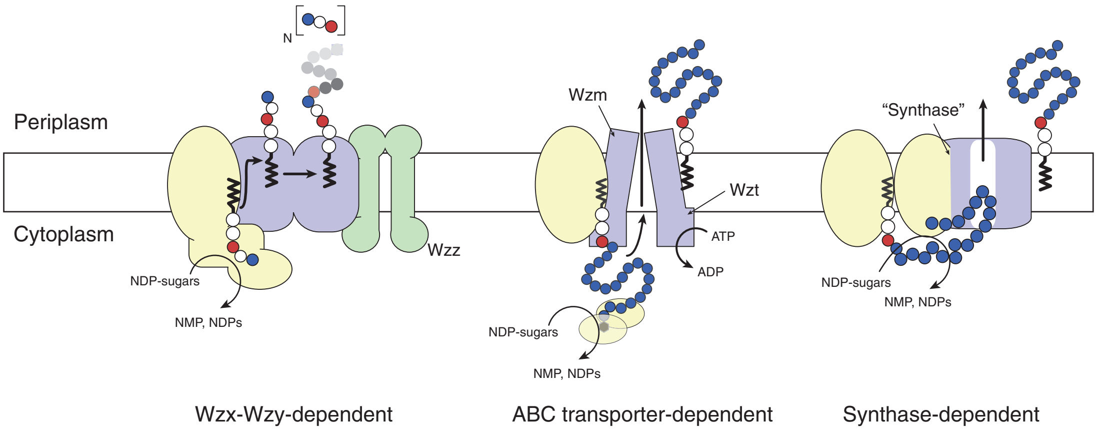
The essential requirement for lipid A and the overall conservation of the Raetz pathway in most bacteria affords opportunities for therapeutic intervention. To date, the most promising target is the zinc-dependent deacetylase, LpxC. LpxC shares no confounding similarity with mammalian deacetylases or amidases. Early LpxC inhibitors were hydroxamate derivatives that interact with the catalytic zinc ion. However, subtle differences in LpxC structure meant that while *E. coli* was susceptible, *Pseudomonas aeruginosa* (an important clinical target) was not. A recently developed inhibitor (CHIR-090) shows more promise with broad-spectrum activity and efficacy against *E. coli* and *P. aeruginosa*, which is comparable to that of commercial antibiotics such as ciprofloxacin or tobramycin. A structure has been solved for LpxC from *Aquifex aeolicus* in complex with CHIR-090 and this represents a major step in further refinements of inhibitors.

The completed Kdo$_2$-lipid A provides an acceptor for glycosyltransferases that act sequentially to assemble the core OS as well as those that add the oligosaccharide chains of LOSs. Assignments of glycosyltransferases that synthesize core OS and enzymes such as kinases that modify the backbone have primarily been made by approaches where specific genes are individually mutated and the resulting LPS structure is resolved by chemical analysis. In some cases, these mutations have complex effects since they alter the structure of the acceptors for additional enzymes, that is, a single mutation can give a phenotype suggesting multiple defects. For some enzymes detailed biochemical properties and solved structures are now available.

Synthesis of O-Polysaccharides

Despite the diversity in O-PS structures, only three mechanisms are known for their formation of O-PS (**Figure 7**). O-PS synthesis begins at the cytoplasmic face of the inner membrane with activated precursors (sugar nucleotides, nucleotide diphosphosugars) and the process terminates with a nascent O-PS at the periplasmic face. Und-P is an obligatory carrier in all three O-PS assembly pathways. The involvement of a carrier lipid scaffold may ensure fidelity in O-repeat unit structure, or simply provide an acceptor compatible with the membrane environment. Interestingly, the ligase from *E. coli* K-12 will ligate structurally distinct O-PSs formed by any of the three assembly pathways, indicating that the form in which nascent O-PS is presented for ligation is conserved. WaaL is the only known component involved in ligation but other factors seem to be involved in the molecular recognition of core OS acceptors and the underpinning mechanism is unknown.

The three pathways for assembly of O-antigens vary in the components required for polymerization, in the cellular location of the polymerization reaction, and in the manner in which material is exported across the inner membrane (**Figure 7**). The same three mechanisms are identified in the biosynthesis of capsular polysaccharides



Wzx-Wzy-dependent ABC transporter-dependent Synthase-dependent

Figure 7 The three known pathways for the biosynthesis of O-polysaccharides (O-PS). All of the assembly systems begin at the cytoplasmic face of the inner membrane and assemble the nascent O-PS in an undecaprenyl pyrophosphoryl (und-PP)-linked form. Nucleotide diphosphosugars (NDP-sugars) are the activated precursors. The enzyme complexes include integral and peripheral proteins, and key components are indicated by name. The pathways differ in the mechanism and location of O-PS polymerization, and in the manner by which O-PS (or O-repeat units) are transferred across the plasma membrane. Termination of O-PS synthesis occurs with ligation of the nascent O-PS to preformed lipid A-core OS at the periplasmic face of the membrane (see **Figure 5**).

in Gram-negative and Gram-positive bacteria. In this respect, the primary distinction between the O-PSs and capsular polysaccharides is that the O-PSs are attached to lipid A-core.

The earliest known pathway for O-PS synthesis is distinguished by the involvement of the putative 'O-PS polymerase' enzyme, Wzy. The 'Wzy-dependent' system (**Figure 7**) is the classical pathway first described in *S. enterica* serogroups A, B, D, and E. Sequence and biochemical data show that the key enzymes (Wzx and Wzy) are shared by other bacteria. In the working model for this pathway, und-PP-linked O-repeat units are assembled by glycosyltransferase enzymes at the cytoplasmic face of the inner membrane. These reactions have been known since work in the 1960s by H. Nikaido, M. J. Osborn, P. Robbins, A. Wright, and others. The initial transferase is an integral membrane protein that transfers sugar-1-phosphate to the und-P acceptor. This is followed by sequential sugar transfers, catalyzed by additional peripheral glycosyltransferases, to form an und-PP-linked repeat unit. The polymerization reaction occurs at the periplasmic face of the membrane and utilizes und-PP-linked O-PS repeat units as the substrate. The individual und-PP-linked O-PS repeat units must, therefore, be exported across the inner membrane prior to polymerization, and preliminary biochemical analyses suggest that the likely candidate for this process is a multiple membrane-spanning protein, Wzx. Wzx has specificity for the first sugar in the und-PP-linked repeat unit. Polymerization of the O-PS repeat units minimally involves the putative polymerase (Wzy) and the O-PS chain length regulator (Wzz), and genetic data suggest critical interactions between Wzx, Wzy, and Wzz. Polymerization occurs in a block-wise process where the growing glycan is transferred from its lipid carrier to the newly exported und-PP-linked repeat unit. A *wzy* mutant is unable to polymerize O-PS and its LPS comprises a single O-repeat unit attached to lipid A-core (sometimes called 'semirough'-LPS). In contrast, a *wzz* mutant makes S-LPS but loses the characteristic modal distribution (**Figure 2**) of O-PS chain lengths evident in SDS-PAGE analysis. The O-PSs synthesized by this pathway are all heteropolymers and often have branched repeating unit structures. An interesting feature in some bacterial species is the presence of additional O-antigenic determinants whose addition is encoded by lysogenic bacteriophage. As an example, *Shigella* O-PS is modified by acetyl and glucosyl residues at different sites in the O-repeat unit using genes provided by prophages. These modifications are added to und-PP-linked O-PS intermediates at the periplasmic face of the inner membrane. The glucosyl donor is und-P-Glc.

In the 'ABC transporter-dependent' pathway (**Figure 7**) the O-PS is synthesized exclusively inside the cytoplasm and once complete, it is exported to the periplasm via an ABC transporter. The transporter is comprised of two copies each of an integral membrane protein, Wzm, and its nucleotide-binding partner, Wzt. As with the Wzy-dependent pathway, synthesis is initiated at the cytoplasmic face of the inner membrane by an integral membrane glycosyltransferase enzyme, to form an und-PP-sugar. In fact, in *E. coli* the UDP-GlcNAc:undecaprenyl phosphate GlcNAc-1-phosphate transferase (WecA) can initiate for either pathway. However, in the ABC transporter-dependent pathway the initiating transferase acts only once per chain, rather than once per repeat unit. Additional peripheral glycosyltransferases then act sequentially and processively to elongate the und-PP-linked intermediate at the nonreducing terminus to form a fully polymerized und-PP-O-PS. In the established prototype for this system, the chain length is regulated by addition of a terminating residue to the growing glycan. The termination residue also provides an essential export signal that is recognized by a structure-specific binding domain located on the nucleotide-binding protein of the ABC transporter. The mechanism by which an ABC transporter can translocate a long chain O-PS linked to und-PP across the cytoplasmic membrane is intriguing but unresolved.

The 'synthase-dependent' pathway for O-PS biosynthesis is, so far, confined to the one homopolymeric O-antigen (factor 54) of *S. enterica* serovar borreze. The model for this pathway (**Figure 7**) proposes that the initiating glycosyltransferase (WecA in this case) forms an und-PP-sugar acceptor that is elongated by a single multifunctional synthase enzyme, in a manner analogous to eukaryotic chitin and cellulose synthases, and chondroitin/hyaluronan synthases from eukaryotes and bacteria. There is no dedicated ABC transporter or Wzx homologue in the O:54 system and all experimental evidence currently points to the 'synthase' having dual transferase-export functions.

Functions and Biological Activities of Lipopolysaccharides

Role of LPS in the Outer Membrane Integrity

In *E. coli* and other closely related enteric bacteria, there are estimated to be approximately 10^6 LPS molecules per cell, encompassing nearly three quarters of the total outer cell surface. The distinct structural features of LPS allow the outer membrane to function as a selective barrier, preventing entry of many toxic molecules and allowing the cell to survive in different environments. A limited number of wild-type Gram-negative bacteria are viable without LPS; this phenomenon was first seen in meningococci where compensatory changes in outer membrane phospholipids and surface lipoproteins may help maintain viability. In the case of *S. paucimobilis*, no 'typical' LPS molecule is present in the outer membrane; instead, the bacterium produces glycosphingolipid, a modified ceramide derivative containing glucuronic acid and an attached trisaccharide. The smallest naturally occurring LPS molecule is lipid IV_A in *F. tularensis* and the intracellular growth environment for this organism, together with other features of the cell envelope, facilitates its survival in the absence of more complex LPS structure.

Outer membrane asymmetry (i.e., LPS in the outer leaflet and glycerophospholipids in the inner leaflet) is essential to the barrier properties. When this is not achieved, and glycerophospholipids migrate to the outer leaflet, the resulting areas of the outer membrane become freely permeable to large hydrophobic antibiotics that normally affect only Gram-positive bacteria, detergents (e.g., SDS), and bile salts (sodium cholate and deoxycholate). Although *E. coli* can assemble an outer membrane from lipid IV_A, the barrier function of the resulting outer membrane is severely compromised and the mutants are highly susceptible to hydrophobic compounds. Because of the reaction sequence in *E. coli* (**Figure 6**), lipid IV_A lacks both Kdo moieties and secondary acyl chains; both influence outer membrane properties. It has been suggested that the Kdo moiety helps stabilize the lipid bilayer by participating in divalent cation bridges formed between negative charges contributed by both the phosphorylated lipid A backbone and the carboxyl group of Kdo. These ionic bridges not only minimize electrostatic repulsion but also promote strong lateral interactions between neighboring LPS molecules. Addition of the secondary acyl chains to lipid IV_A has been implicated in maintaining a low degree of fluidity, a condition that is critical to outer membrane function. Although secondary acyl chains are not essential for viability, under-acylation of lipid A often results in growth defects. The normal tight packing of saturated acyl chains induces a network of hydrophobic interactions that help maintain the integrity of the outer leaflet of the outer membrane through van der Waal's forces.

In many bacteria, the core OS also contributes to outer membrane integrity. In *E. coli* and *Salmonella*, the phosphorylated inner core Hep residues (**Figure 4**) are particularly important. Their absence leads to a pleiotropic-defective envelope phenotype called 'deep rough' that is reflected in hypersensitivity to hydrophobic compounds, such as detergents, dyes, and some antibiotics, as well as other physiological changes. *Salmonella waaP* mutants are avirulent. In *P. aeruginosa*, phosphorylation of the Hep region is essential for viability. These modifications may provide further avenues for therapeutic intervention. The importance of negatively charged core OS residues in a robust outer membrane is underscored by *K. pneumoniae* where the phosphates are absent and carboxyl groups provided by GalA residues are important.

O-Polysaccharides as a Protective Barrier

Molecular modeling of LPS structure and its organization in the outer membrane suggest that the O-PS partially lies flat on the cell surface, where the crossover of multiple chains forms a 'felt-like' network. Since the O-PS is flexible, it can also extend a significant distance from the surface of the outer membrane and cryo-electron microscopy reveals a significant O-PS layer on the cell surface. It is therefore not surprising that many properties attributed to the O-PS are protective.

Long-chain O-PS is typically essential for resistance to complement-mediated serum killing and, therefore, represents a major virulence factor in many Gram-negative bacteria. The serum proteins in the complement pathway interact to form a membrane attack complex (MAC) that can integrate into lipid bilayers to produce pores, leading to cell death. The MAC can be formed via a 'classical' pathway, where surface antigen–antibody complexes initiate MAC formation, or through the 'alternative' pathway, where complement component C3b directly interacts with the cell surface in the absence of antibody to facilitate MAC formation. In Gram-negative bacteria with S-LPS, resistance to the alternative pathway does not result from defects in C3b deposition. Instead, C3b is preferentially deposited on the longest O-PS chains, and the resulting MAC is unable to insert into the outer membrane. In addition to O-PS chain length, complement resistance can also be influenced by the extent of coverage of the available lipid A-core with O-PS. As is often the case, there are exceptions to such generalizations. For example, there are some *E. coli* strains with S-LPS that are serum-sensitive unless an additional capsular polysaccharide layer is present. Although R-LPS variants of *E. coli* and *Salmonella* are almost invariably serum-sensitive, other bacteria (including many with LOS) use alternate strategies to achieve resistance.

The bactericidal/permeability-inducing protein (BPI) is an antibacterial product found in polymorphonuclear leukocyte-rich inflammatory exudates. BPI binds LPS and plays a role in the clearance of circulating LPS (see 'Lipopolysaccharide and Gram-negative sepsis'), but it also exhibits antimicrobial activity in the presence of serum. Resistance to BPI-mediated killing is also dependent on long-chain O-PS.

Lipopolysaccharide and Gram-Negative Sepsis

One potential outcome of Gram-negative infection is septic shock, a syndrome manifest by hypotension, coagulopathy, and organ failure. In the United States, Gram-negative sepsis accounts for nearly 200 000 deaths each year. LPS (endotoxin) is responsible for this effect but it is now clear that it does not act as a 'toxin' in its classical sense. Instead, septic shock results from the interaction of LPS molecules released from bacteria with macrophages and endothelial cells, leading to the unregulated host production of cytokines and inflammatory mediators, including tumor necrosis factor-α (TNF-α) and a variety of interleukins. Under normal circumstances, these components have beneficial effects and lead to moderate fever, general stimulation of the immune system, and microbial killing. However, their unregulated overproduction leads to tissue and vascular damage and the symptoms of sepsis. Since free LPS is required to initiate the process, treatment with antibiotics and the ensuing bacterial lysis can actually exacerbate the problem.

The last decade has seen significant advances in our understanding of the manner in which LPS interacts with animal cells and stimulates their production of mediator molecules and the structural principles of endotoxity. It is the structure of lipid A that dictates the biological (endotoxic) properties. Some partial LPS structures and chemically synthesized lipid A derivatives display the same biological activities as the complete molecule. Importantly, other partial structures (including lipid IV_A) and some naturally occurring LPS (e.g., the product from *Rhodopseudomonas sphaeroides*) are biologically inactive and can act as antagonists, negating the biological activity of 'toxic' LPS. These structures have directed the synthesis of potent synthetic LPS antagonists. Many of the structural principles underlying the biological activity have been resolved; the phosphates and the position and types of acyl chains are critical. LPS molecules form micellar aggregates. Their adopted shape is dictated by packing of LPS monomers (a function of their structure) and is a key determinant in their endotoxic properties. The prototypical lipid A structure of *E. coli*, containing two phosphate groups and six acyl chains consisting of 12–14 carbons, has maximal biological activity. In contrast, lipid A molecules that show low biological activity typically possess only four or five acyl chains, some which are 16–18 carbons in length, and they contain only one phosphate group attached to lipid A.

Many elements of the complex system by which cells respond to LPS have now been identified (**Figure 8**). Circulating LPS aggregates interact with a variety of host proteins, which are important in mobilizing LPS monomers from such complexes; these proteins include BPI and LBP, an acute phase protein produced by hepatocytes. One role of these proteins is to clear and detoxify LPS. For example, LBP is known to transfer LPS to high-density lipoprotein fractions. However, LBP is also a crucial component of the signaling pathway through which animal cells are stimulated to produce cytokines and inflammatory mediators. The central pathway by which cells recognize low concentrations of LPS (or bacterial envelope fragments containing LPS) requires the participation of a receptor protein CD14; CD14-knockout mice are 10 000-fold less sensitive to LPS *in vivo*. In myeloid cell lines, CD14 occurs as a glycosylphosphatidylinositol (GPI)-anchored glycoprotein attached to the membrane CD14 (mCD14). However, a variety of non-myeloid (endothelial and epithelial) cells are also responsive to LPS via a soluble form of CD14 (sCD14). Both sCD14 and mCD14 can bind LPS to form a complex, but the kinetics of binding is slow. LBP serves to overcome this rate-limiting step by delivering LPS to mCD14 or sCD14. CD14 has a hydrophobic pocket that accommodates the acyl chains of lipid A. This protects the molecule from the enzymatic activity of acyloxyacyl hydrolase, a leukocyte enzyme whose function is to selectively remove the secondary (acyloxyacyl-linked) fatty acyl chains from lipid A, thereby detoxifying the molecule.

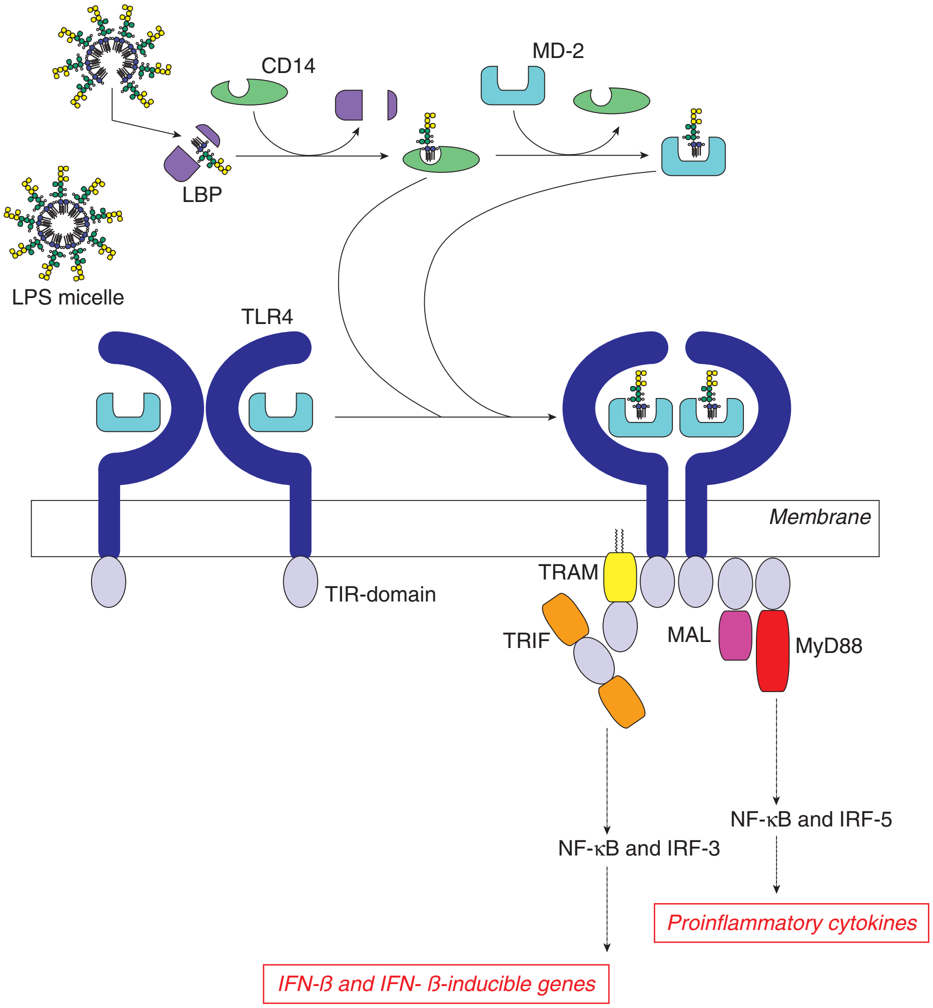


Figure 8 The pathway involved in the stimulation of macrophages and other myeloid cell lines by LPS. The LPS-binding protein (LBP) binds to Gram-negative bacteria or aggregated LPS and delivers an LPS molecule to CD14 and this is transferred to MD-2. The binding of MD-2:LPS to TLR4 causes TLR4 to dimerize and leads to the productive association of the intracellular TIR domains on the TLR4 monomers. This in turn facilitates the recruitment of adaptor proteins MAL/MyD88 and TRAM/TRIF to activate the MyD88-dependent and MyD88-independent pathways. In each case, a multifactorial signal transduction cascade leads to transcription of genes encoding cytokines and inflammatory mediators. Some elements of the pathway are also important for LPS clearing.

Although CD14 is a central element in the signaling pathway, it has no transmembrane domain to facilitate intracellular signaling, nor can it discriminate between agonist and antagonist lipid A species. Thus an accessory coreceptor was hypothesized but its identity remained elusive for some time. Major breakthroughs in understanding the process came with the discovery of the central roles played by a membrane protein, Toll-like receptor 4 (TLR4), and a secreted glycoprotein called MD-2 (myeloid differentiation-2). Both MD-2 and TLR are essential for LPS responsiveness. MD-2 serves as an adaptor linking the extracellular recognition and intracellular signaling events. MD-2 exists in two forms; one is soluble and the other is membrane-bound through an association with TLR4. MD-2 binds LPS and apparently recognizes both acyl chains and lipid A phosphates. It is likely that MD-2 recognizes structural features of different LPS molecules and confers species-specific recognition on TLR4 reflected in agonist/antagonist responses and signals of differing strength. For example, the lipid A molecules from *Helicobacter pylori*, *Yersinia pestis*, *F. tularensis*, *Legionella pneumophila*, *Porphyromonas gingivalis*, and *Chlamydia trachomatis* all have much lower biological activities. The potential of these pathogens to cause severe disease in human is attributed, in part, to their ability to avoid TLR4 signaling. An essential step in the signaling process is the delivery of LPS to the tethered MD-2 and this is likely achieved by either soluble MD-2 or CD14. However, the processes are unclear and some reports also implicate intercalation of LPS aggregates in the cell membrane as part of the signaling pathway. The engagement of tethered MD-2 with LPS results in signal transfer across the cell membrane, through a mechanism that involves dimerization of TLR4. Crystal structures for TLR4:MD-2 and MD-2:lipid A have been reported. TLR4 dimerization facilitates the productive association of intracellular TIR (Toll/interleukin I receptor) domains on each monomer and initiates the recruitment of additional cytoplasmic adaptor proteins that also contain TIR domains.

These adaptor proteins include MyD88 (myeloid differentiation factor 88), MAL (MyD88 adaptor-like protein), TRIF (TIR domain-containing adaptor inducing IFNβ, also known as TICAM-1), and TRAM (TRIF-related adaptor molecule). These adaptors act in concert with a complex array of additional positive and negative regulators to control the activation of IRF (interferon regulatory factor) proteins and NFκB, leading to expression of different sets of genes. MAL is membrane-associated and recruits MyD88 to activate the MyD88-dependent signaling pathway. In contrast, membrane-associated TRAM recruits TRIF to stimulate the MyD88-independent pathway. These pathways play an important role in the proflammatory response as well as in the development of LPS tolerance.

A variety of therapeutic approaches have been designed to interfere with specific steps in the process leading to septic shock. The number of mediators involved complicates strategies based on blocking cytokines themselves; for example, antibody neutralization of TNF-α is not effective. Significant efforts have been directed to neutralizing the LPS using antiendotoxin monoclonal antibodies, but the results have been disappointing. However, antibodies that recognize the core OS show promise in preliminary studies. LPS neutralization could be achieved by using proteins that bind LPS, and both LBP and BPI are being pursued in that respect. Other strategies meeting success in early-phase studies include attempts to block the extracellular steps in LPS receptor/signaling pathways. The approaches include delivery of synthetic LPS antagonist(s), application of anti-CD14 and anti-LBP monoclonal antibodies that block the formation of CD14/LPS complex, or administration of antibodies against MD-2 and TLR4 to inhibit signal transfer.

Lipid A Modification Systems

Modification of lipid A can contribute in a very significant way to the virulence and pathogenic capabilities of many Gram-negative bacteria, by modulating interaction with the innate host defenses. These modifications can allow the bacterium to evade recognition by TLR4 as well as promoting resistance to host cationic antimicrobial peptides (CAMPs). The modifications are often induced by environmental signals and occur after lipid A transport across the inner membrane by MsbA; the modifying enzymes are located at the periplasmic face of the inner membrane or in the outer membrane. This separation of lipid A biosynthesis from latent modifications may be important to maintain efficient synthesis of lipid A and to ensure proper transport across the inner membrane. The potential modifications are well illustrated using *Salmonella* LPS as an example.

In the laboratory, the basal *Salmonella* LPS structure contains a hexa-acylated lipid A with phosphates at positions 1 and 4′. In contrast, in the presence of the appropriate environmental signals, the lipid A is heavily decorated and is hepta-acylated (**Figure 9**). The lipid A modifications are governed by two different two-component regulatory systems, PhoP/PhQ and PmrA/B. PhoP/PhoQ is required for transcription of genes essential for virulence in mice and humans; it is important for survival within macrophages and for resistance to CAMPs. PhoP/PhoQ is activated after phagocytosis by macrophages and signals to the bacterium its presence inside the phagolysosome, which contains a variety of antimicrobial peptides and other surface-active compounds. In the laboratory, the PhoP/PhoQ system can be activated under low Mg^{2+} conditions and by exposure of the bacterium to CAMPs. In *Salmonella*, activation PhoP/PhoQ also initiates the PmrA/B pathway via a posttranslational mechanism using an effector protein known as PmrD. However, in *E. coli* the *pmrD* gene is nonfunctional.

Following activation via PhoP/PhoQ, the acyl chains of *Salmonella* lipid A are extensively remodeled. PagP is a palmitoyltransferase that transfers palmitate from glycerophospholipids to lipid A to form a hepta-acylated species with an acyloxyacyl derivative at position 2. This addition may afford tighter packing of LPS. PagP is important for resistance to CAMPs, and PagP homologues are essential for virulence in many Gram-negative pathogens. PagL and LpxR (which is Ca^{2+}-regulated) are deacetylases whose roles are unclear; both are latent in *Salmonella* and their influence on LPS structure is evident only when the enzymes are overexpressed. In *Salmonella*, PagL is not required for resistance to CAMPs. LpxR homologues are widespread, and are apparently active, in other pathogens (e.g., *H. pylori*, *Yersinia enterocolitica*, and *P. gingivalis*) where ester-linked acyl chains are absent from the 3-*O* and 3′-*O* positions. Modification of *Salmonella* LPS by either PagP or PagL is sufficient to attenuate signaling through TLR4 and may serve to dampen host response (inflammation) in infected hosts.

Modification to the disaccharide region centers on the removal or decoration of the lipid A phosphate groups. *Salmonella* contains latent enzymes capable of modifying the lipid A phosphates with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or PEtN (**Figure 9**) following activation of PmrA/PmrB. Substitution of the 1- and 4′-phosphates with L-Ara4N and PEtN helps to mask the negatively charged lipid A phosphate groups. This lowers the affinity of lipid A for CAMPs and polycationic compounds such as

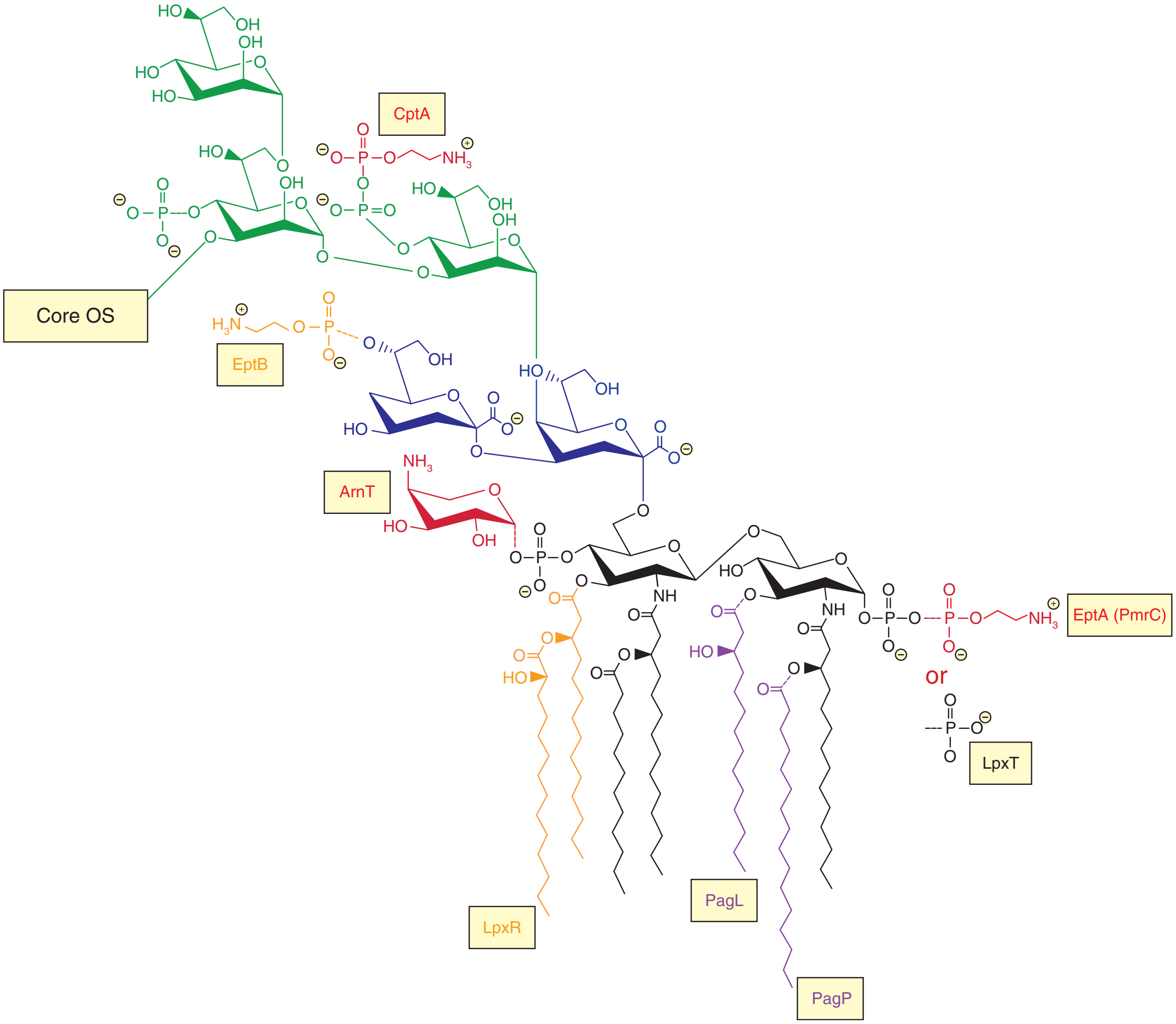


Figure 9 Regulated covalent modifications of *Salmonella enterica* serovar Typhimurium lipid A-inner core. The base lipid A structure is indicated in black, Kdo residues in blue, and phosphorylated Hep residues in green. PhoP/PhoQ-regulated modifications influencing acylation are shown in purple and PmrA/PmrB-regulated modifications affecting charge are shown in red. The modifications in orange are regulated by the Ca^{2+} content of the medium.

polymyxin (the Pmr name is derived from 'polymyxin resistance'), and therefore prevents a critical step in the killing of bacteria by CAMPs. Bacteria harboring the modified LPS survive longer inside neutrophils. The transfer of L-Ara4N to lipid A occurs at the periplasmic face of the inner membrane and is catalyzed by ArnT, using und-P-Ara4N as a donor, in a reaction resembling phage-mediated glucosylation of O-PS (see above). The *arn* genes are well distributed. PEtN is transferred from phosphatidylethanolamine by the inner membrane enzyme EptA (also known as PmrC). In *Salmonella*, addition of Ara4N appears more important than addition of PEtN in CAMP-resistance. In contrast, *N. meningitidis* cannot add Ara4N and its natural resistance to CAMPs is conferred by its EptA homologue. In *H. pylori*, the 1-phosphate on the lipid A is removed prior to addition of PEtN. In *E. coli*, the 1-phosphate can be modified with an additional phosphate group which is transferred from und-PP by LpxT. The 1-diphosphorylated lipid A species in *Salmonella* are identified only in Pmr-null mutants lacking Ara4N and PEtN. The nature of the regulatory process that balances these modifications, and the impact of the 1-diphosphate, have not been established. Other modifications of the backbone phosphates include methyl groups in *Leptospira interrogans*, galactosamine residues in *F. tularensis*, and GalA in some *Rhizobium* species.

In *Salmonella*, the core OS is also subject to modification with PEtN (**Figure 9**). Modifications of Kdo and Hep-P are catalyzed by EptB (Ca^{2+}-regulated) and CptA (PmrA/PmrB-regulated), respectively; both may be required for outer membrane stability under certain conditions.

Rather than masking the negatively charged phosphate groups, some bacteria adopt an alternative strategy in which the lipid A phosphate groups are removed enzymatically. Gram-negative bacteria whose lipid A lacks one or more phosphates include *H. pylori*, *P. gingivalis*, *Bacteroides*, *L. interrogans*, and others. Removal of the phosphate groups reduces the endotoxic property of lipid A, while at the same time reducing its affinity for antimicrobial peptides. The influence of this modification is illustrated by expression of a 1-phosphatase gene (*lpxE*) from *Sinorhizobium* in *Salmonella*. The resulting LPS is a nonendotoxic adjuvant and the resulting strain may be useful in development of live oral vaccines.

Perhaps the most radical postsynthesis modification is seen in *F. tularensis*, where free lipid A can be found on the cell surface. It is proposed that this is derived from nascent S-LPS through the action of one (or more) Kdo-hydrolase(s). This is accompanied by removal of the 4′-phosphate (by LpxF), 3′-deacylation and addition of GalN to the 1-phosphate, by a homologue of ArnT.

Phase Variation and Molecular Mimicry in LPS and LOS

Pathogenic bacteria have developed a wide array of strategies to overcome the host immune system for extended survival in their host. Gram-negative bacteria often use phase variation, a reversible change in antigenic determinants including LPS. The mechanisms involved in on–off switching of target genes include genomic rearrangements, slipped-strand mispairing, or variations mediated by differential methylation that change the expression of specific genes. Phase variation of LPS is prevalent and well studied in mucosal pathogens with LOS such as *Neisseria* spp., *Haemophilus influenzae*, and in *H. pylori* and *Campylobacter jejuni* it generates a complex mixture of glycoforms. The resulting structures are subject to intense selective pressures in the host and can significantly influence host–pathogens. For example, the LPS of *Neisseria gonorrhoeae* isolates typically contain low amounts of sialic acid, and this is important for entry of the bacterium into human mucosal epithelial cells. In contrast, variants with highly sialylated LPS resist killing by complement and antibodies but fail to enter into human mucosal epithelial cells. Although not a classical phase variation process, *P. aeruginosa* isolates colonizing the pulmonary system of cystic fibrosis patients undergo a gradual change in the LPS phenotype involving loss of the long-chain anionic O-PS known as B band. This host adaptation appears to be a regulated development, since on subsequent culture *in vitro*, cells revert to typical S-LPS profiles.

Molecular mimicry is also seen frequently in LPS and LOS. For example, the O-chain of *H. pylori* LPS contains fucosylated OS and Lewis antigen epitopes, which mimic human blood group antigens. Variable expression of these epitopes influences evasion of host immune response, autoimmune response, cell adhesion, and long-term colonization of the bacteria. The LOS of *C. jejuni* and *Brucella melitensis* contain phase-variable ganglioside mimics and these may again provide a mechanism for avoiding the host immune response. However, they are also implicated in the development of an autoimmune response underlying Guillain–Barré syndrome.

Biotechnological Applications Involving LPS

The incredible array of LPS structures provides an extensive range of oligosaccharide and polysaccharide structures with novel biological properties. These may be of value for therapeutic or other commercial applications. In one novel example, a recombinant *E. coli* strain was constructed in which the LPS core OS provided a scaffold for expression of the globotriose receptor for Shiga toxin. The surface-exposed receptor in the *E. coli* strain efficiently adsorbs and neutralizes the toxin, affording a therapeutic approach to treating infections whose pathogenesis involves this and related toxins. Another exciting example is the recent development of monophosphoryl lipid A (MPL) as a vaccine adjuvant. The effectiveness of LPS as an immunomodulator is well known; however, its extreme toxicity precludes its therapeutic use. MPL is a chemically modified derivative of *Salmonella* LPS that displays greatly reduced toxicity, while maintaining most of the immunostimulatory activity of LPS. MPL adjuvant has been extensively used in clinical trials as a component in prophylactic and therapeutic vaccines targeting infectious disease, cancer, and allergies. Structures of glycosyltransferases, including an LOS galactosyltransferase, have been solved at high resolution by crystallographic methods. Ultimately, this will provide insight into the details of structure–function relationships among glycosyltransferases and open the possibility for engineering enzymes with novel specificities for practical applications. For information regarding known glycosyltransferases see the CAZY (Carbohydrate Active enZYmes) website maintained by B. Henrissat's laboratory (http://www.cazy.org/).

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Relevant Websites

http://www.cazy.org – Carbohydrate active enzymes (CAZY)

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Marine Habitats

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Defining Statement
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Nature of Marine Microbial Life
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Further Reading

Glossary

autotroph An organism that uses carbon dioxide as its source of carbon for new cell growth. Autotrophs can be either phototrophs or chemotrophs.

chemotroph An organisms that derives its energy from reduced inorganic or reduced organic compounds.

chlorophyll A ubiquitous pigment that is responsible for light energy absorption in the photosynthetic apparatus of most marine phototrophs.

euphotic zone The sunlit portion of the water column where there is a sufficient light flux to sustain net photosynthesis, usually the upper 150–200 m in the clearest ocean water.

genome The complement of genes present in a living organism that determines its taxonomic structure, metabolic characteristics, behavior, and ecological function.

habitat A place of residence that is defined by a suite of physical, chemical, and biological characteristics.

mixotrophy A term used to describe the metabolism of a microorganism that can obtain energy, electrons, or carbon (or all three) from more than one conventional source.

nutrient One of several organic or inorganic raw materials that are used by microorganisms to sustain their metabolism, for example, nitrate, phosphate, iron, vitamins.

phototroph An organism that derives its energy from sunlight, usually through the process of photosynthesis.

picoplankton Small (0.2–2 μm in diameter) phototrophic, chemotrophic, or mixotrophic organisms that live in the water column and drift with the ocean currents.

remote sensing The indirect measurement of habitat characteristics, for example, by Earth-orbiting satellites.

turbulence A physical process resulting from wind stress, ocean circulation, and related processes that is responsible for the exchange of heat and mass between two or more regions of the ocean, for example, the transport of nutrients from the deep sea to the sunlit surface waters.

twilight zone The region of the oceanic realm (also called the mesopelagic zone) immediately below the euphotic zone where sunlight is measurable but insufficient to support net photosynthesis, usually between 200 and 1000 m.

Abbreviations

ALOHA	A Long-term Oligotrophic Habitat Assessment
ATP	adenosine triphosphate
BATS	Bermuda Atlantic Time-series Study
CZCS	Coastal Zone Color Scanner
DCML	Deep Chlorophyll Maximum Layer
DOM	dissolved organic matter
DON	dissolved organic nitrogen
HOT	Hawaii Ocean Time-series
HTL	higher trophic level
MODIS	Moderate Resolution Imaging Spectroradiometer
NPSG	North Pacific Subtropical Gyre
OSP	Ocean Station Papa
PAR	Photosynthetically Available Radiation
SEATS	SouthEast Asia Time-Series
WOCE	World Ocean Circulation Experiment

Defining Statement

A habitat is the natural abode of an organism. The marine habitat is composed of a diverse spectrum of environments each supporting the proliferation of a diverse assemblage of microorganisms. When habitats vary, for example as a result of seasonal and longer term climate forcing, the diversity and function of the microbial assemblage will also change. The North Pacific Subtropical Gyre (NPSG), one of Earth's largest habitats, is an excellent example of a marine habitat in motion with respect to microbial structure and function.

Introduction

A habitat is often defined as the natural abode or place of residence. For this reason, the global ocean may be considered as one of the largest and oldest habitats on Earth; it covers 71% of the Earth's surface to a mean depth of 3.8 kilometers and comprises >95% of Earth's probable living space (**Figure 1**). However, despite the appearance of homogeneity, the ocean is actually a complex mosaic of many different macrohabitats that can be identified, studied, and compared; each macrohabitat has a potentially distinct assemblage of microorganisms. Examples are rocky intertidal, coastal upwelling, deep sea hydrothermal vent, and open ocean habitats.

Some of these habitats are critical to the mass balance of elements in the ocean because they are found at the interface or boundary between terrestrial, or freshwater, and marine systems. For instance, within estuarine habitats, which are located at the freshwater–marine boundary, processes can trap dissolved nutrients and particulate matter, or can export energy in the form of organic matter and nutrients to surrounding coastal habitats. The characteristics of any given estuary will vary depending upon dimensions, hydrology, and geographical location. And, because they represent gradual transition zones, estuaries highlight the difficulty of defining the boundaries of marine habitats. The continental–marine boundary also includes many specialized and important microbial habitats including fjords, salt marshes, mangrove stands, coral reefs, kelp forests, and many man-made or human-impacted (e.g., harbors, sewage outfalls, mariculture farms, gas and oil production facilities) zones.

Another approach to define a marine habitat is through the comprehensive list of physical, chemical, and biological parameters experienced directly by the organism during its lifetime; collectively these parameters determine the success or failure of a particular strain, species, or assemblage of microbes. Given this definition, the size, motility, and

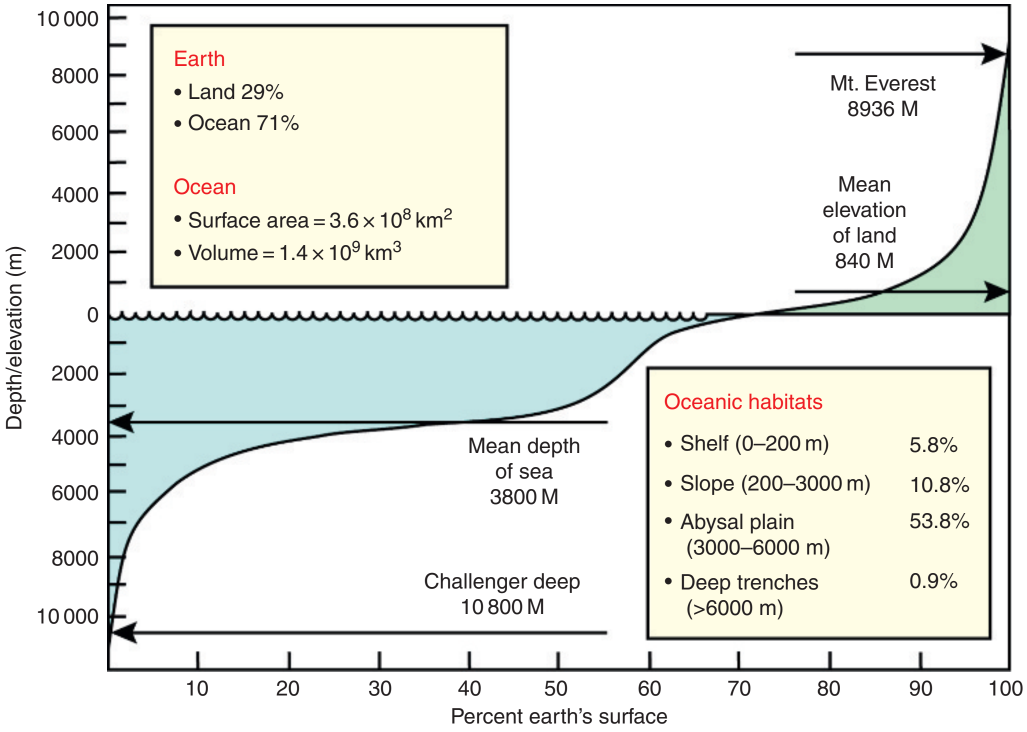

Figure 1 The world ocean covers 71% of Earth's surface with the deep blue sea (regions seaward of the continental shelf) accounting for more than 60% of the total. With an average depth of 3800 m, the volume of habitable space on planet Earth is dominated by marine habitats. Because microbes dominate the marine environment, they are directly or indirectly linked to most global processes and are largely responsible for the habitability of our planet. Reproduced, with permission, from *Microbial Oceanography* (http://www.agi.org).

lifespan of the microbes under consideration define the spatial and temporal scale of their habitat. Hence, it is important to recognize that most microorganisms live in 'microhabitats' that are defined on space scales of micrometers to millimeters and time scales of hours to weeks.

Microhabitats are often the sites of elevated microbial biomass and accelerated metabolism. Once colonized by microorganisms, the environmental conditions within a given microhabitat (e.g., pH, redox level, nutrients, and dissolved gas concentrations) can change as a result of the metabolic activities of the microbial assemblage. Consequently, a description of the macroscopic surrounding habitat (scales of meters to kilometers) may not always be a valid representation of the true habitat of existence. This is the reason why otherwise incompatible microbes, for example, obligate aerobic and anaerobic microorganisms can co-occur in a single environmental sample. Therefore, any given marine habitat – particularly when viewed on macroscale (meter or more) – is likely to be composed of numerous microhabitats that collectively support the growth and proliferation of the microbial assemblage as a whole.

Historically, there has been a concerted effort to define only the physical/chemical properties of a given marine habitat, but more recently the key role of biotic factors in establishing and maintaining microbial community structure and function has been recognized. Many microorganisms in the sea live attached to surfaces including nonliving particulate matter and living organisms. These surfaces help to create and sustain the above-mentioned microhabitats; at the same time they provide local enrichments of organic matter. In addition, bacteria and other microbes can also be found within the digestive tracts of marine macroorganisms, from small crustaceans to large cetaceans; it is likely that every macroorganism in the sea has a unique, species-specific set of microbial partners that are not commonly found elsewhere. And, because many marine macroorganisms (e.g., tuna, squid, and seabirds) have broad geographical distributions and enormous migratory capability, these animal vectors may affect the geographic distribution range of their microbial partners. Additional biotic interactions include virus–host interactions, gene exchange between otherwise unrelated microorganisms, microbe–microbe and microbe–macroorganism symbioses, obligate metabolic partnerships (syntrophy), and coevolution of different groups of microorganisms.

At a microbial level, for many syntrophic relationships to succeed there needs to be a high probability of juxtaposition of cell types to maximize interspecific interactions. This can be achieved in a homogeneous environment where microbes live freely but in close proximity, or through an ordered spatial structure of the microbial assemblage as a whole. In marine sediments and other 'solid' habitats, for example, microbial communities are often established along diffusion gradients with each cell type growing in the most favorable microhabitat within the gradient in order to maximize success. Growth along these stable gradients, where stability is defined as a function of microbial generation time, can lead to the development of microbial (usually bacterial) mats. Analogous features termed microbial lens or plates can occur in highly stratified 'fluid' habitats, for example, at the oxic–anoxic boundaries of permanently anoxic basins such as the Black Sea or Cariaco Basin. Metabolic activities of microorganisms in these mats and lens can be very high, thereby producing extremely steep vertical gradients of biomass and other metabolic by-products.

Even though we know that microbes are by far the largest contributors to living matter in the sea and have been responsible for the development of the atmosphere under which terrestrial life evolved, much of the research on the role of the habitat in structuring marine microbial communities and their ecosystem function is fairly new, incomplete and lacking any formal theoretical description or predictive capability under changing habitat conditions. And, because seascapes are changing, in part, due to the activities of human populations, a comprehensive understanding of sea microbes and their activities under various global environmental change scenarios is a major and urgent intellectual challenge.

Nature of Marine Microbial Life

Life on Earth most likely began in the sea; so the marine environment was the original habitat for the growth and proliferation of microorganisms. As the pioneering prokaryotes evolved into more complex life forms, including multicellular macroscopic organisms, and radiated into freshwater habitats and eventually onto land, the imprint of a marine origin remained. Today, virtually all life is intimately dependent upon the availability of water; even desert microbes are aquatic.

Aquatic habitats are built around the unique properties of water. The most important criterion is the fact that water is a polar molecule having positively and negatively charged sides. This characteristic establishes its high dielectric constant and effectiveness as a solvent, setting the stage for the high dissolved ionic (salt) composition of seawater (referred to as salinity). During the HMS *Challenger* expedition of 1872–1876, the 'law of constant proportions' was confirmed, namely that the ratios of the major ions in seawater are relatively constant throughout the world ocean. This relative ionic stability has very important implications for the evolution of marine microorganisms. Furthermore, the unique solvation property of water also facilitates nutrient delivery to and waste material export from the cell, thereby sustaining microbial metabolism.

Other water properties including density and gas solubility, which vary with temperature and salinity, can also have major implications for the distribution and abundance of microorganisms. The density of pure water at 4 °C is 1.000 g cm^{-3}, decreasing slightly to 0.994 g cm^{-3} at 35 °C; the average density of seawater is ~1.025 at 25 °C (in marine sciences, the density is often expressed as an anomaly ((density − 1.000) × 1000], to amplify the small differences in density between freshwater and seawater; for example, the density anomaly of average seawater would be ((1.025 − 1.000) × 1000] or 25.0). This means that river and rain water will float on seawater, as will surface waters warmed by the sun, whereas colder and saltier seawater will sink. As a result, the world's ocean is highly stratified with depth; mass exchange and transport occur mainly within layers of constant density (along isopycnals) or via turbulent mixing of waters with different densities (across isopycnals). Diffusional exchange processes that depend on molecular kinetic energy are, by comparison, very slow. This vertical density stratification, a hallmark of the marine environment, tends to insulate inorganic nutrient-rich deep seawaters from the sunlit surface region where the capture of solar energy by biological systems (photosynthesis) occurs. Consequently, density stratification strongly influences the rate of organic matter production and attendant ecosystem services.

In order for marine organisms to live in the water column they need to remain in suspension. The specific gravity of marine microbes varies with their bulk chemical composition (e.g., protein = 1.5 g cm^{-3}, nucleic acids = 2.0 g cm^{-3}, lipids = 0.9 g cm^{-3}) which is, in turn, dependent upon nutrient supply, growth rate, and other biotic factors. If the mean cell density is less than the density of seawater, the cells will tend to rise. Conversely, if the bulk cell density is greater than seawater or if microbes are attached to dense particulate materials, cells will settle. Many marine microorganisms adjust their density by the formation (or collapse) of gas vacuoles, alterations in the ionic composition of the cytoplasm, or by adjusting the above-mentioned composition of the cell, for example, by the synthesis of storage components such as carbohydrates that can serve as ballast. The rate at which cells will rise or settle is also related to their size causing small cells to remain suspended in their environment. Furthermore, most microorganisms, even small bacteria, are motile by means of one or more flagella, but movement through a relatively viscous medium at low Reynold's numbers can be difficult (the Reynold's number is a dimensionless metric that determines whether inertial or viscous forces dominate motion of an object in a fluid). Small bacteria and virus particles are also displaced by means of Brownian motion, a process driven by the random movement of water molecules that can act as a counterforce to gravitational settling. However, even though many marine microorganisms in the water column are motile, their directed movements are small relative to the marine currents in which they reside. Hence, they drift with the currents and are commonly known as plankton (from the Greek root *Plankto*, which means 'wandering').

In addition to its role in density stratification, solar radiation provides most of the energy required to fuel the biological activity in marine environments. Water has a characteristic solar absorption spectrum that allows electromagnetic radiation between ~350 and 700 nm to penetrate to various depths in the water column depending upon surface solar irradiance, sun angle, and water clarity; the euphotic zone is generally defined as the water column region located above a specific isolume (a constant daily level of irradiance) or above a specific percentage (usually 1 or 0.1%) of the surface irradiance. The water absorption spectrum, with a maximum transmission at 417 nm, creates a sharp gradient, in terms of both the intensity and the quality of light available as a function of depth. For example, while photosynthetic organisms confined to surface waters are exposed to high light intensities within a broad spectrum range within the visible (400–700 nm) and extending into the near-ultraviolet region (350–400 nm), deeper marine habitats experience lower light intensities due to the exponential decrease of light with depth and a shift toward a dominance of blue light. Hence, while organisms in surface waters have had to adapt to protect themselves against excess light and ultraviolet radiation by producing photoprotective pigments, organisms living near the base of the euphotic zone require strategies that increase their capabilities of solar energy capture in the blue region of the spectrum; this is achieved by increasing the number of photosynthetic units per cell and by modifying the spectral absorption characteristics through changes in the photosynthetic pigments associated with them. As a consequence, the light gradient can generate and sustain a highly structured vertical pattern of light-harvesting microorganisms in the marine environment; some microbes are adapted to high and others to lower light fluxes. In addition, the surface light intensity and its propagation through the water column define the region where the photosynthetic rates of the microbial assemblage can exceed its respiration, driving the balance between the uptake (photosynthesis) and remineralization (respiration) of nutrients with depth. It is this balance in microbial activity that ultimately drives the biological sequestration and transport of elements, such as carbon, in the ocean and defines the large-scale distribution and availability of nutrients in the marine environment.

Structure and Classification of Marine Macrohabitats

There are numerous criteria that can be used to classify marine habitats. The most widely accepted scheme divides the ocean into two broad categories: pelagic and benthic,

Table 1 Classification of marine habitats according to Hedgpeth (1957)

Region	*Boundaries/comments*
Pelagic Realm (water column)	
Neritic	Waters over the continental shelves (~200 m)
Oceanic	Waters seaward of the continental shelves
- epipelagic	0–200 m; sunlit regions
- mesopelagic	200–1500 m
- bathypelagic	1500–4000 m
- abyssopelagic	>4000 m
Benthic Realm (seabed)	
Supralittoral	Above high-tide mark
Littoral	Between the tides
Sublittoral	Between low tide and edge of continental shelves (~200 m)
Bathyal	Seaward of continental shelves to ~4000 m
Abyssal	4000–6000 m
Hadal	>6000 m, including trenches

depending upon whether the habitat of interest is the overlying water column (fluid) or the seabed (solid), respectively (**Figure 1**; **Table 1**). Within each of these main categories, a number of additional subdivisions can be made depending, for example, on increasing water depth from the high tide mark. For the pelagic habitat, major subdivisions include neritic for waters overlying the continental shelves ($\leq$200 m deep) and oceanic, for the vast open sea. The oceanic realm can also be further subdivided (**Table 1**). Benthic habitats include littoral (intertidal), sublittoral (from the low tide boundary to edge of continental shelf), bathyal, abyssal, and hadal. Other classification schemes use the topographic boundaries: continental shelf, continental slope, abyssal plain, and deep sea trenches (see **Figure 1**). Although these and other terms are routinely used, the depth ranges are not always identical, so they should be considered as guidelines rather than rules.

Along the seawater depth gradient, whether in benthic or pelagic habitats, some physical and chemical characteristics systematically change (e.g., decreasing temperature and increasing pressure). In this regard, it is important to emphasize that the most common marine habitat is cold (<12 °C) and exposed to high hydrostatic pressure (>50 bars). Consequently, many marine microbes are cold- and pressure-adapted, even obligately so; indeed, some abyssopelagic bacteria require high pressure (>400 bars) to grow. Other classification schemes based on the availability of sunlight (euphotic (light present) or aphotic (light absent)) or the relative rates of organic matter production (eutrophic (high), mesotrophic (medium), or oligotrophic (low)) have also been used.

Sharp horizontal gradients can also be observed in the surface of the ocean. For example, since the 1960s, oceanographers have used satellite-based remote sensing approaches to map various features of the global ocean, including sea surface temperature, winds, altimetry, and the distributions of photosynthetic microbes as inferred from observations of spectral radiance. The first satellite-based ocean color measurements were obtained using the Coastal Zone Color Scanner (CZCS) aboard the Nimbus-7 satellite that was launched in October 1978; it provided useful data for nearly a decade. The CZCS sensor was eventually replaced with the Sea-viewing Wide Field-of-view Sensor (SeaWiFS), launched in September 1997, and still operational, followed by Moderate Resolution Imaging Spectroradiometer (MODIS) aboard the Terra and Aqua satellites (1999 and 2002 to present, respectively). And, although these instruments cannot provide information regarding spatial variability below 1 km resolution, they have provided unprecedented observations on the temporal variability or surface ocean macrohabitats (depth-integrated to one optical depth, ~25 m in clear open ocean waters), as well as the mesoscale (10–100 s of km) and large-scale distributions of chlorophyll (chl). Daily synoptic global images can be pieced together to track the dynamics (days to decades) of photosynthetic microbial assemblages in the global ocean and their correlations with other environmental variables in ways that are not possible by any other means (**Figure 2**). Furthermore, systematic analyses of these ocean color datasets can be used to define spatial habitat structure in oceanic ecosystems, and the partitioning of the global ocean into a suite of ecological provinces or functional habitat units, leading to the novel subdiscipline of marine ecological geography. Unfortunately, there are no satellite-based sensors that can track non-chl-containing marine microbes, although several novel remote detection systems are under development for *in situ* application based on molecular/genetic probes and imaging-in-flow cytometry.

There are distinct water types throughout the ocean that can be easily identified by measuring their temperature and salinity characteristics, which together determine their densities (T-S diagram; **Figure 3**). Using T-S diagrams as a fingerprinting tool, water types can be traced throughout the world ocean to specific regions of formation. A water mass results from the mixing of two or more water types, and is represented by a line between distinct water types on the T-S diagram (**Figure 3**). Water masses can also be tracked for great distances throughout the world ocean, and their microbial assemblages can also be sampled and characterized. The global circulation rate, as deduced by nonconservative chemical properties and radioisotopic tracers, has a time scale of hundreds to thousands of years. Consequently, 'young' and 'old' water masses can be identified based on the time that the water was last in contact with the atmosphere.

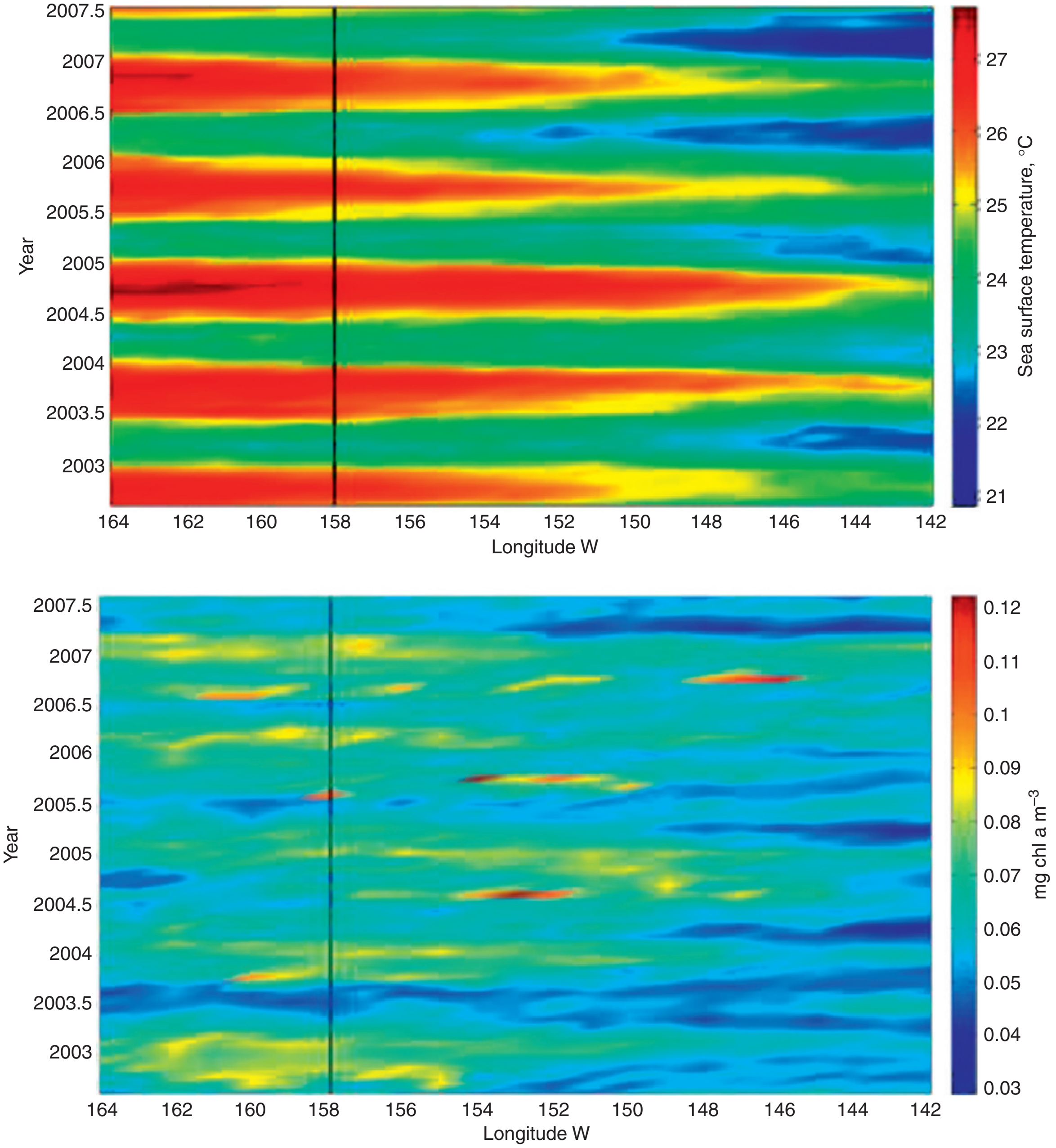

Figure 2 Satellite-derived temporal and longitudinal variability in sea surface temperature (Top) and chlorophyll (Bottom) for the region surrounding Station ALOHA (22.75°N, 158°W). The data, available from the NASA Ocean Color Time-Series Online Visualization and Analysis website (http://reason.gsfc.nasa.gov/), have been obtained through NASA's Moderate Resolution Imaging Spectroradiomater (MODIS) sensor on board Aqua between July 2002 and June 2007 and correspond to the latitudinal average between 22.5°N and 23.5°N for the longitude band 142–164°W. The black line marks longitude 158°W where Station ALOHA is located.

Due to unique seafloor topography and interactions with the atmosphere, certain regions 'short-circuit' the mean circulation by serving as conduits for a more rapid ventilation of the deep ocean (bringing it into contact with the atmosphere) and the concomitant delivery of nutrient-rich deep water to the surface of the sea. These so-called upwelling regions occupy only ~1% of the surface ocean, but they are important areas of solar energy capture through enhanced photosynthesis and the selection of relatively large algae and short food chains; thereby they support some of the great fisheries of the world (**Figure 4**). In contrast, the more common condition (90% of the global ocean) is the oligotrophic habitat (low nutrient and low rates of organic

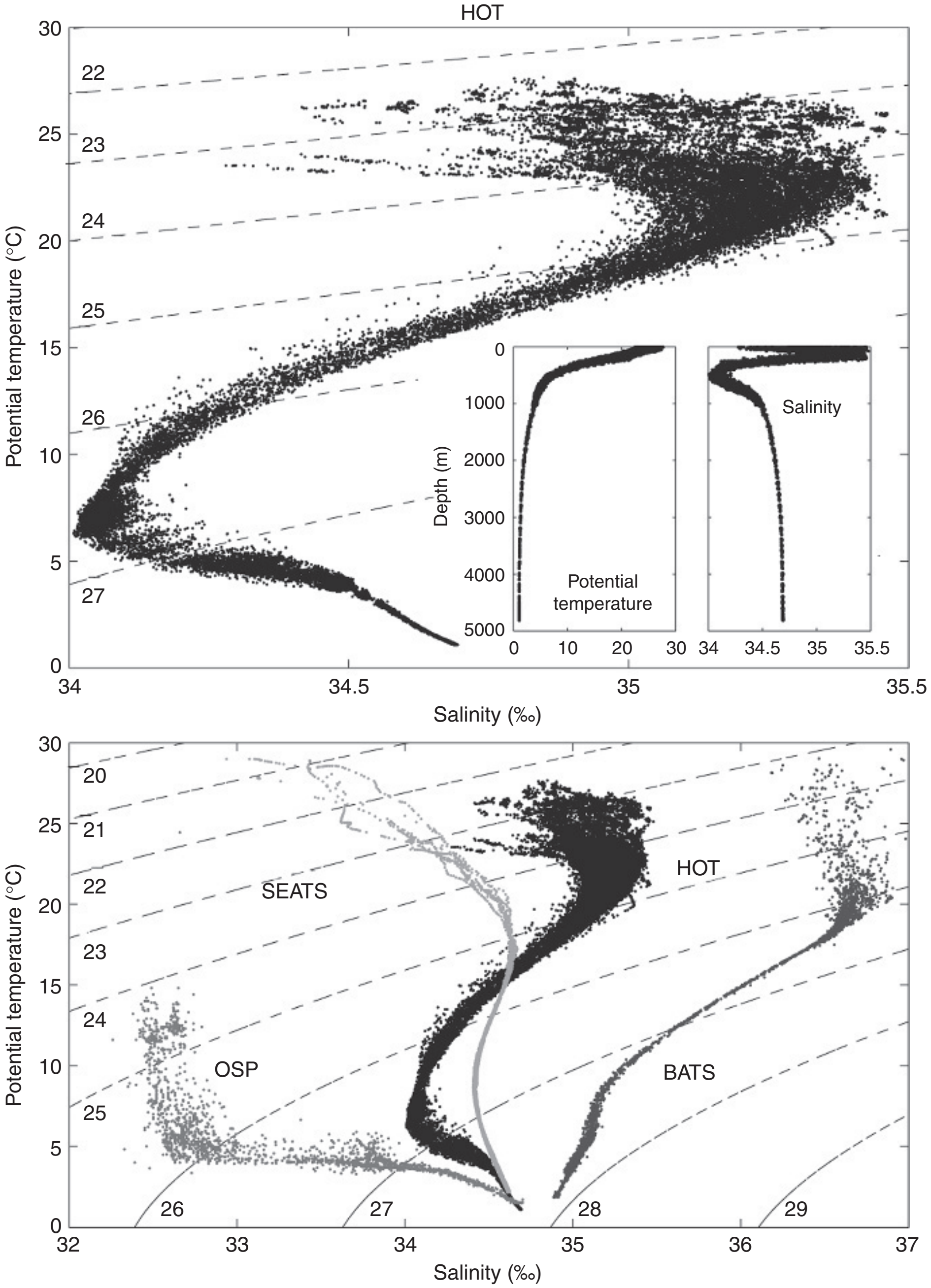

Figure 3 Potential temperature versus salinity (T-S) plots are used to identify, trace, and compare distinct water types and water masses in the marine environment. (Top) T-S diagram for the Hawaii Ocean Time-series (HOT) Station ALOHA for the period 1988–2006. The inset shows the depth profiles of potential temperature and salinity. The ALOHA T-S fingerprint shows the presence of numerous water masses at specific depths. The contours show lines of constant density, or isopycnal surfaces, in density anomaly notation ((density in g cm^{-3})$-$1.000)$\times$1000). In addition to temperature and salinity (density) variations, these distinctive water masses also have distinctive chemical properties and may contain unique assemblages of microorganisms. The large variability of T and S at the top of the graph is a result of seasonal and interannual changes in near-surface water properties. (Bottom) Comparison of T-S fingerprints for a variety of oceanic time-series stations including: Ocean Station Papa (OSP; 50°N, 145°W), SouthEast Asia Time-Series (SEATS; 18°N, 116°E), Hawaii Ocean Time-series (HOT; 22.75°N, 158°W), and Bermuda Atlantic Time-series Study (BATS; 32°N, 64°W). The three North Pacific stations (OSP, SEATS, HOT) have a common deep water mass.

matter production) that selects for very small primary producers, long and complex microbial-based food webs, and relatively inefficient transfer of carbon and energy to higher trophic levels like fish. These fundamental differences in physics result in marine habitats with diverse structures and dynamics that host dramatically different microbial assemblages, as discussed later in this article.

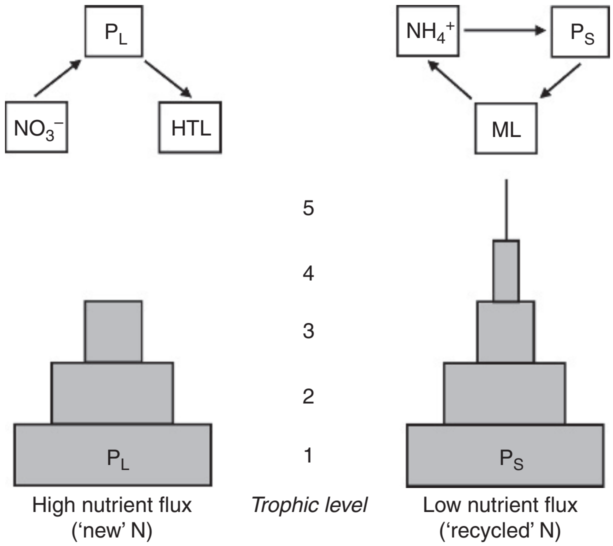

Figure 4 Importance of nutrient flux on the size distribution and efficiency of biomass and energy flow in marine habitats. The schematic on the left depicts a habitat where 'new' nutrient (as NO_3^-) flux is high (e.g., an upwelling region). This leads to a selection for large phytoplankton cells (P_L) that are efficiently consumed by higher trophic levels (HTLs) including large zooplankton and fish. This results in a short and efficient food chain. In contrast to the upwelling regions, most open ocean habitats have low new nutrient (NO_3^-) fluxes and survive by local remineralization of required nutrients ('recycled'). These conditions select for small phytoplankton cells (P_S) that serve as the food source for long and complex microbial-based food webs (also called microbial loops; ML) that recycle mass and dissipate most of the solar energy that was initially captured. The great marine fisheries of the world are generally found in association with upwelling regions.

Marine Microbial Inhabitants and Their Growth Requirements

The marine environment supports the growth of a diverse assemblage of microbes from all three domains of life: *Bacteria*, *Archaea*, and *Eucarya*. The term 'microorganism' is a catchall term to describe unicellular and multicellular organisms that are smaller than $\sim 100-150\,\mu\text{m}$. This grouping includes organisms with broadly distinct evolutionary histories, physiological capabilities, and ecological niches. The only common, shared features are their size and a high surface-to-biovolume ratio. A consequence of being small is a high rate of metabolism and shorter generation times than most larger organisms.

Microorganisms, particularly bacteria and archaea, are found throughout the world ocean including marine sedimentary and subseabed habitats. There is probably no marine habitat that is devoid of microorganisms, with the possible exception of high-temperature (>100 °C) zones. In addition to a physically favorable environment, the metabolism and proliferation of microorganisms also require a renewable supply of energy, electrons for energy generation, carbon (and related bioelements including nitrogen, phosphorus, sulfur), and occasionally organic growth factors such as vitamins. Depending upon how these requirements are met, all living organisms can be classified into one of several metabolic categories (**Table 2**). For example, photolithoautotrophic microbes use light as an energy source, water as an electron source, and inorganic carbon, mineral nutrients, and trace metals to produce organic matter. At the other end of the metabolic spectrum, chemoorganoheterotrophic microbes use preformed organic matter for energy generation and as a source of electrons and carbon for cell growth. In a laboratory setting, only obligate photolithoautotrophs are self-sufficient; all other autotrophs and all heterotrophs rely upon the metabolic activities of other microorganisms. However in nature, even obligate photolithoautotrophs must tie their growth and survival to other, mostly deep-sea, microbes that are vital in sustaining nutrient availability over evolutionary time scales. Most marine microorganisms probably use a variety of metabolic strategies, perhaps simultaneously, to survive in nature. Because needed nutrients in the ocean's surface are often found in dissolved organic molecules, it seems highly improbable that sunlit marine habitats would select for obligate photolithoautotrophy as opposed to, for instance, mixotrophic growth.

Table 2 Variations in microbial metabolism based on sources of energy, electrons, and carbon according to Karl (2007)

Source of energy[a]	*Source of electrons*	*Source of carbon*
Sunlight	Inorganic	CO_2
photo-	*-litho-*	*-autotroph*
	Organic	Organic
	-organo-	*-heterotroph*
Chemical	Inorganic	CO_2
chemo-	*-litho-*	*-autotroph*
	Organic	Organic
	-organo-	*-heterotroph*
Radioactive decay	Inorganic	CO_2
radio-	*-litho-*	*-autotroph*
	Organic	Organic
	-organo-	*-heterotroph*

[a] A 'mixotroph' is an organism that uses more than one source of energy, electrons, or carbon.

Across the full metabolic spectrum of possible modes of growth, some microbes are more self-sufficient than others. For example, while most microbes require a supply of chemically 'fixed' nitrogen, either in reduced (ammonia or dissolved organic nitrogen (DON)) or in oxidized (nitrate or nitrite) form to survive, a special group of N_2-fixing microbes (diazotrophs) can use the nearly unlimited supply of dissolved N_2 as their sole source of cell N. Additionally, some microbes can manufacture all their required building blocks (e.g., amino acids and nucleic acid bases) and growth cofactors (e.g., vitamins) from simple inorganic precursors, whereas others require that they be supplied from the environment; 'auxotrophic' microorganisms are, therefore, ultimately dependent upon the metabolic and biosynthetic activities of other microbes. These 'incomplete' microbes, probably the bulk of the total microbial assemblage in seawater, cannot grow unless the obligate growth factors are present in and resupplied to the local habitat. In this regard, most marine habitats provide the laboratory equivalent of a complex or complete medium containing low-molecular-weight compounds (e.g., amino acids, simple sugars, nucleic acid bases, and vitamins), in addition to the mineral nutrients and trace metals. The active salvage and utilization of these biosynthetic precursors, in lieu of *de novo* synthesis, conserves energy, increases growth efficiency, and enhances survival. Over evolutionary time, some unused biosynthetic pathways in particular organisms appear to have been lost from the genome, perhaps, as a competitive strategy for survival in a mostly energy-limited environment. This process has been termed genome streamlining.

Finally, growth and reproduction are often viewed as the most successful stages of existence for any microorganism. However, in many of the low nutrient concentration and low energy flux habitats that dominate the global seascape, the ability to survive for extended periods under conditions of starvation may also be of great selective advantage and ultimately may affect the stability and resilience of microbial ecosystems. The starvation-survival response in marine bacteria leads to fragmentation (i.e., cell division in the absence of net growth) and, ultimately, to the formation of multiple dwarf or miniaturized cells. Other physiological changes, including reduction in endogenous metabolism, decreases in intracellular adenosine triphosphate (ATP) concentrations, and enhanced rates of adhesion are also common consequences. These starved cells can respond rapidly to the addition of organic nutrients. This 'feast and famine' cycle has important implications for how we design *in situ* metabolic detection systems and model microbial growth in marine habitats.

Distribution, Abundance, and Biogeography of Marine Microbes

The distribution and abundance of microbes is highly variable, but somewhat predictable, across globally distributed marine habitats. For example, phototrophic microbes are restricted to sunlit regions (0–200 m in the open sea) whereas chemotrophic microbes are found throughout the oceanic realm. However, because the abundance and productivity of marine microbes depend on the availability of nutrients and energy, there is often a decreasing gradient in total microbial biomass from the continents to the open ocean, and a decreasing gradient in total microbial biomass from the sunlit surface waters to the abyss. For the pelagic zone, total microbial biomass in near-surface (0–100 m) waters ranges from 30 to 100 mg carbon m^{-3} in neritic waters to 6–20 mg carbon m^{-3} in oceanic waters. For open ocean habitats, this biomass decreases by approximately three orders of magnitude from euphotic zone to abyssal habitats, with values <0.02 mg carbon m^{-3} in the deepest ocean trenches. When scaling these concentrations to the volume of the ocean, the total oceanic microbial biomass, excluding sediments, has been estimated to be $0.6-1.9 \times 10^{15}$ g carbon with approximately half its stock residing below 100 m.

Temperature is an important habitat variable, and may be responsible for structuring microbial assemblages and setting limits on various metabolic processes. However, temperature *per se* does not limit the existence of marine microbes so long as liquid water exists. Accordingly, there are some marine habitats that select for thermophilic microbes ('warm temperature-loving'; e.g., deep-sea hydrothermal vents) and others for psychrophilic microbes ('cold temperature-loving'; e.g., polar latitudes and abyssal regions). Spatial gradients in temperature across open ocean habitats as well as seasonal changes in temperature can also affect the diversity of microbial assemblages in most marine habitats. Finally, for any given microbial species, there is a positive correlation between rates of metabolism and temperature over its permissive range. Generally, for a 10 °C change in temperature there is a two- to threefold increase in metabolic activity, for example, respiration. Photochemical reactions, including photosynthesis, have much smaller temperature coefficients, and it has been hypothesized that low temperature suppression of chemoorganoheterotrophic bacterial activity, relative to photosynthesis, might significantly restrict energy flow through microbial food webs, increasing the efficiency of the transfer of carbon and energy to higher trophic levels via metazoan grazing. This is just one way in which temperature may structure and control microbial processes in the sea.

In the near-surface waters, microbes capture solar energy, which is locally transferred and dissipated as heat, or exported to other surrounding marine habitats in the form of reduced organic or inorganic substrates, including biomass. Apart from very restricted shallow coastal regions where light can penetrate all the way to the seabed for use by benthic micro- and macroalgae, essentially all marine photosynthesis is planktonic (free floating) and microbial. The dynamic range in total marine photosynthesis, from the most productive to the least productive regions of the global ocean, is probably less than two orders of magnitude for a given latitude, and the biomass of chemoorganoheterotrophic bacteria may be even less. There are much steeper gradients in photosynthesis and bacterial/archaeal biomass in the vertical (depth) than with horizontal (spatial) dimensions. Furthermore, most marine respiration is also driven by microbes, both phototrophs and chemotrophs. For this reason, the mean turnover time of oceanic carbon within biological systems in surface waters is weeks, compared to decades for most terrestrial ecosystems.

Size spectral models and analyses, which relate the relative abundance of organisms as a function of size, have been used to examine the distribution of biomass among various size classes. The emergent patterns from these analyses, particularly between and among different marine habitats, are relevant to issues regarding the environmental controls on microbial community structure and function as well as to the trophic efficiency of marine food webs. In some oceanic habitats, solar energy is captured and completely utilized within microbial-based food webs; in other regions a significant proportion of the energy captured via photosynthesis is passed to large organisms, including fish and humans. An important consideration appears to be the size of the primary producer populations, and this determines the number of trophic transfers that are sustainable in light of the typically inefficient (<10%) transfer of carbon and energy between trophic levels (**Figure 4**). If, for example, the primary producers are relatively large (>10 –20 μm diameter; P_L in **Figure 4**, left), such as unicellular algae including diatoms, rather than tiny picoplankton (<2 μm; P_S in **Figure 4**, right), then the grazer/consumer based food chain is shorter, leading to a more efficient transfer of carbon and energy (**Figure 4**). However, the length of the food chain is not always defined by the difference in size between the primary producer and the top consumer; in some cases, large organisms such as baleen whales have adapted a feeding strategy that relies mostly on very small, planktonic organisms. Nevertheless, the size and structure of marine food webs is determined, in large part, by physical processes such as turbulence, which, in turn, affects the flux of nutrients into the euphotic zone and, therefore, shapes the structure and function of marine ecosystems.

In most sunlit marine habitats there is generally a significant correlation between chl concentrations and the number of bacterial cells, and between net primary production and bacterial production across a broad range of ecosystems. These empirical relationships suggest that phototrophs and chemotrophs grow in response to common factors (e.g., nutrients, temperature), or that phototrophs produce substrates for the growth of chemotrophs, or vice versa.

In addition to living organisms, virus particles – particularly those capable of infecting specific groups of microorganisms – can exert influence on microbial-based processes. For example, through microbial infection and subsequent lysis, viral activity may directly influence the composition of the microbial assemblage. Furthermore, through the release of dissolved organic matter (DOM) into the marine environment during virus-induced cell lysis, an indirect effect on metabolic activity of the chemotrophic assemblage can occur. Viruses can also facilitate genetic exchange between different microbial strains contributing to the metabolic plasticity of certain microorganisms and the redundancy of some metabolic processes in a given environment. It has been reported that virus particle abundances closely track the abundance of bacteria plus archaea, at least in the water column, with virus-to-prokaryote ratios ranging from 5 to 25, and commonly close to 10. This relationship appears to hold even into the deep sea, suggesting a close ecological linkage throughout the entire marine habitat.

From an ecological perspective, understanding and modeling how microbial assemblages emerge as a result of interaction of physics and biology is a primary goal in microbial oceanography. In this context, the study of the distribution of biodiversity over space and time, also known as biogeography, seeks fundamental information on the controls of speciation, extinction, dispersal and species interactions such as competition. The field of microbial biogeography is just beginning to develop a conceptual framework and analytical tools to examine distribution patterns and to quantify diversity at the ecologically relevant taxonomic scale. For example, recent studies of the marine phototroph *Prochlorococcus* have documented significant intraspecific genomic variability that confers distinct niche specificity including nutrient and light resource partitioning. What appears at one level to be a cosmopolitan species is actually a group of closely related ecotypes (populations within a species that are adapted to a particular set of habitat conditions); the high- and low-light ecotypes have >97% similarity in their 16S ribosomal RNA gene sequences and share a core of 1350 genes, but vary by more than 30% in their total gene content (and genome size; the high- and low-light adapted ecotypes have genome sizes of 1 657 990 bp and 2 410 873 bp, respectively). An assemblage of related *Vibrio splendidus* (>99% 16S RNA identity) sampled from a temperate coastal marine habitat had at least 1000 distinct coexisting genotypes, and bacterial samples collected from the aphotic zone of the North Atlantic Ocean revealed an extremely diverse 'rare biosphere' consisting of thousands of low-abundance populations. The ecological implications of these independent reports of taxonomic diversity are profound; new ecological theory may even be required to build a conceptual framework for our knowledge of marine habitats and their microbial inhabitants.

Sunlight, Nutrients, Turbulence, and the Biological Pump

Of all the environmental variables that collectively define the marine habitat, we single out three – namely, sunlight, nutrients, and turbulence – as perhaps the most critical for the survival of sea microbes. Together, these properties control the magnitude and efficiency of the 'biological pump', a complex series of trophic processes that result in a spatial separation between energy (sunlight) and mass (essential nutrients) throughout the marine environment. In the sunlit regions of most (but not all) marine habitats, nutrients are efficiently assimilated into organic matter, a portion of which is displaced downward in the water column, mostly through gravitational settling. As particles sink through the stratified water column, a portion of the organic matter is oxidized and the essential nutrients are recycled back into the surrounding water masses. Depending upon the depth of remineralization and replenishment to the surface waters by physical processes, these essential nutrients can be sequestered for relatively long periods (>100 yrs). The vertical nutrient profile, for example of nitrate, shows a relative depletion near the surface and enrichment at depth as a result of the biological pump (**Figures 5**(**a**) and **5**(**b**)); regional variations in the depth profiles reflect the combination of changes in the strength and efficiency of the biological pump and the patterns of global ocean circulation (**Figures 5**(**a**) and **6**). The highest nutrient concentrations in deep water can be found in the abyss of the North Pacific, the oldest water mass on Earth. The regeneration of inorganic nutrients requires the oxidation of reduced organic matter, so the concentrations of dissolved oxygen decrease with depth and with age of the water mass as a result of the cumulative effect of microbial metabolism (**Figures 5**(**b**) and **6**).

Turbulence in marine habitats derives from a variety of processes including wind stress on the ocean's surface, ocean circulation, breaking internal waves, and other large-scale motions that can create instabilities, including eddies, in the mean density structure. Turbulence, or eddy diffusion, differs fundamentally from molecular diffusion in that all properties (e.g., heat, salt, nutrients, and dissolved gases) have the same eddy diffusion coefficient; a typical value for horizontal eddy diffusivity in the ocean is $\sim 500\,\mathrm{m^2\,s^{-1}}$, a value that is 10^9 times greater than molecular diffusion. Vertical eddy diffusivity is much lower ($\sim 0.6\text{–}1 \times 10^{-4}\,\mathrm{m^2\,s^{-1}}$) suggesting that the upward flux of nutrients into the euphotic zone is a slower process than movement horizontally in the open ocean. Most near-surface dwelling microbes, particularly phototrophs that are also dependent upon solar energy and are effectively 'trapped' in the euphotic zone habitat, depend on turbulence to deliver deep water nutrients to the sunlit habitat.

In addition to the eddy diffusion of nutrients from the mesopelagic zone, wind stress at the surface and other forces can mix the surface ocean from above. If the near-surface density stratification is weak or if the mixing forces are strong, or both, then a large portion of the euphotic zone can be homogenized; in selected latitude regions the surface mixing layer can extend to 500 m or more, well below the maximum depth of the euphotic zone. These well-mixed environments usually have sufficient nutrients but insufficient light to sustain photosynthesis because the phototrophs are also mixed to great depths, as in some polar habitats during winter months. Following these seasonal deep-mixing events, the ocean begins to stratify due to the absorption of solar radiation in excess of evaporative heat loss. As the wind forcing from winter storms subsides and the intensity of solar radiation increases, a density gradient develops in the upper water column. Phototrophic microorganisms in the euphotic zone gain a favorable niche with respect to both light energy and nutrient concentrations. Depending upon the presence or absence of grazers, this condition results in an increase in phototrophic microbial biomass, a condition referred to as the spring bloom. A comprehensive formulation of the 'vernal blooming of phytoplankton' presented by H. Sverdrup remains a valid representation of this important marine microbial phenomenon.

However, in many portions of the world ocean, particularly in tropical ocean gyres, local forcing due to wind stress is too weak to break down the density stratification, so the nutrient delivery from below the euphotic zone through mixing is not possible. In these oligotrophic regions, the habitat is chronically nutrient-stressed and oftentimes nutrient-limited. Although surface mixed layers can be observed, they rarely penetrate deeper than 100 m. Even within the so-called mixed layer, gradients in chl, nutrients, dissolved gases, and microorganisms can be detected, suggesting that these regions are not always actively mixing. This subtle distinction between a mixing layer, where there is an active vertical transport of physical, chemical, and biological properties, and a mixed layer, which is defined operationally as a layer with weak or no density stratification, has important implications for microbial growth and survival, particularly for phototrophic microorganisms. Consequently, without additional information on mixing dynamics (e.g., a profile of turbulent kinetic energy), the commonly used term mixed layer can be misleading with regard to habitat conditions for microbial growth. The time required to change from a mixing layer to a mixed layer to a density-stratified surface habitat and back again will depend on the habitat of interest.

One approach for distinguishing between a mixing layer and a mixed layer is to measure the near-surface concentrations and temporal dynamics of a short-lived photochemically produced tracer, for example, hydrogen peroxide (H_2O_2). The concentration versus depth profile of H_2O_2 in a mixing layer with a short mixing time scale (≤ 1 h) would be constant because the concentration of photochemically active DOM and average solar energy flux would also be relatively constant. On the other hand, the H_2O_2 concentration profile in a nonmixing (or slowly mixing, turnover >1 day) 'mixed layer' would approximate the shape to the flux of solar energy decreasing exponentially with depth nearly identical to a density-stratified habitat, assuming that the concentration of photosensitive DOM is in excess. It is also possible to use other photochemical reactions to obtain information on vertical mixing rates.

Time Variability of Marine Habitats and Climate Change

Marine habitats vary in both time and space over more than nine orders of magnitude of scale in each dimension. Compared with terrestrial habitats, most marine ecosystems are out of 'direct sight', and, therefore, sparsely observed and grossly undersampled. The discovery and subsequent documentation of the oases of life surrounding hydrothermal vents in the deep sea in 1977 revealed how little we knew about benthic life at that time. Furthermore, because marine life is predominantly microscopic in nature, the temporal and spatial scales affecting microbial processes may be far removed from the scales that our senses are able to perceive. And, due to this physical and sensory remoteness of marine microbial habitats, even today unexpected discoveries about the ocean frontier continue to be made, many of these involving marine microbes.

We have selected the North Pacific Subtropical Gyre (NPSG) for a more detailed presentation of relationships between and among habitat structure, microbial community function and climate. Our choice of the NPSG as an exemplar habitat is based on the existence of the Hawaii Ocean Time-series (HOT) study, a research program that seeks a fundamental understanding of the NPSG habitat. The emergent comprehensive physical, chemical, and biological data sets derived from the HOT benchmark Station ALOHA (A Long-term Oligotrophic Habitat Assessment) is one of the few spanning temporal scales that range from a few hours to almost two decades. More generally, we submit that the sampling and observational components of the HOT program at the deep water Station ALOHA are applicable to other locations that may be representative of key marine habitats.

The NPSG is one of the largest and oldest habitats on our planet; its present boundaries have persisted since the

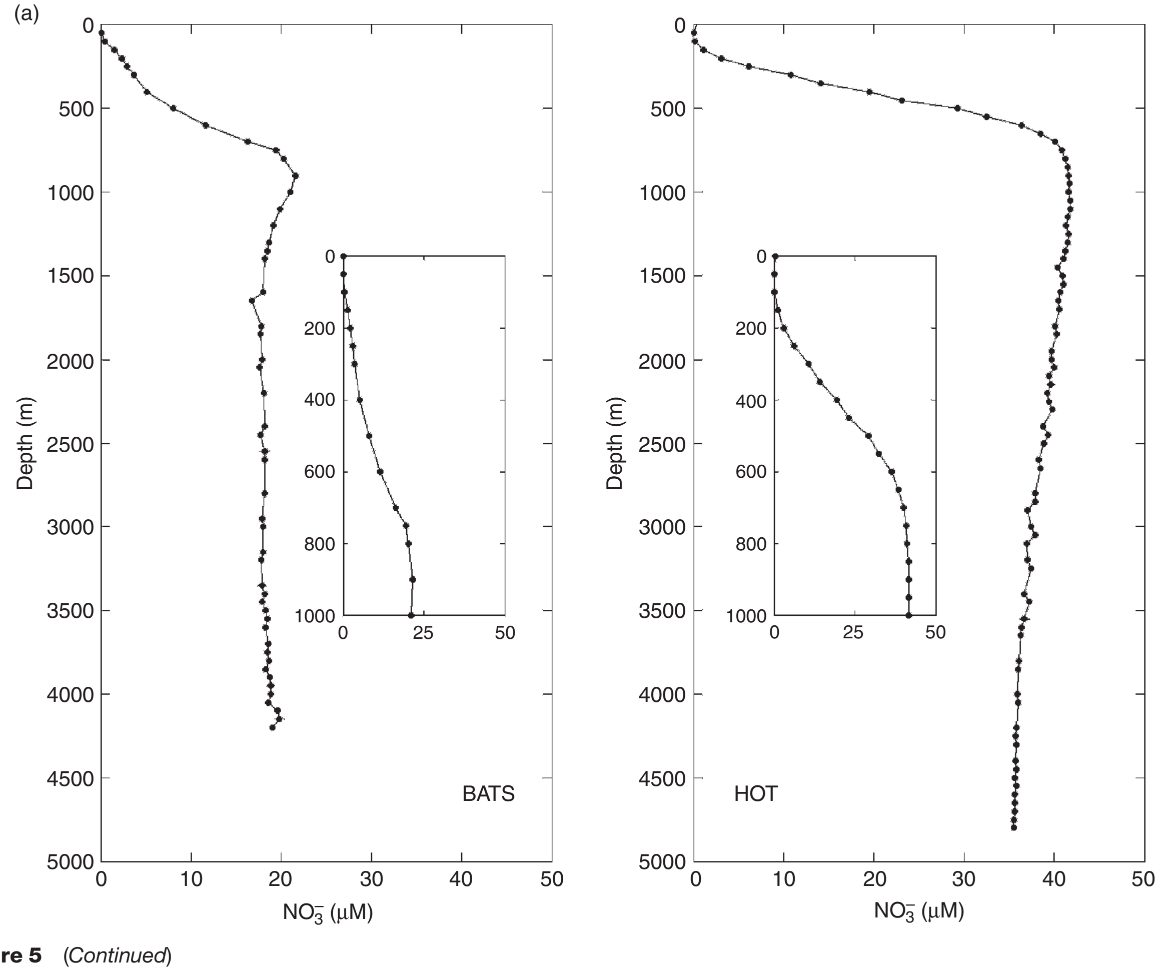

Figure 5 (*Continued*)

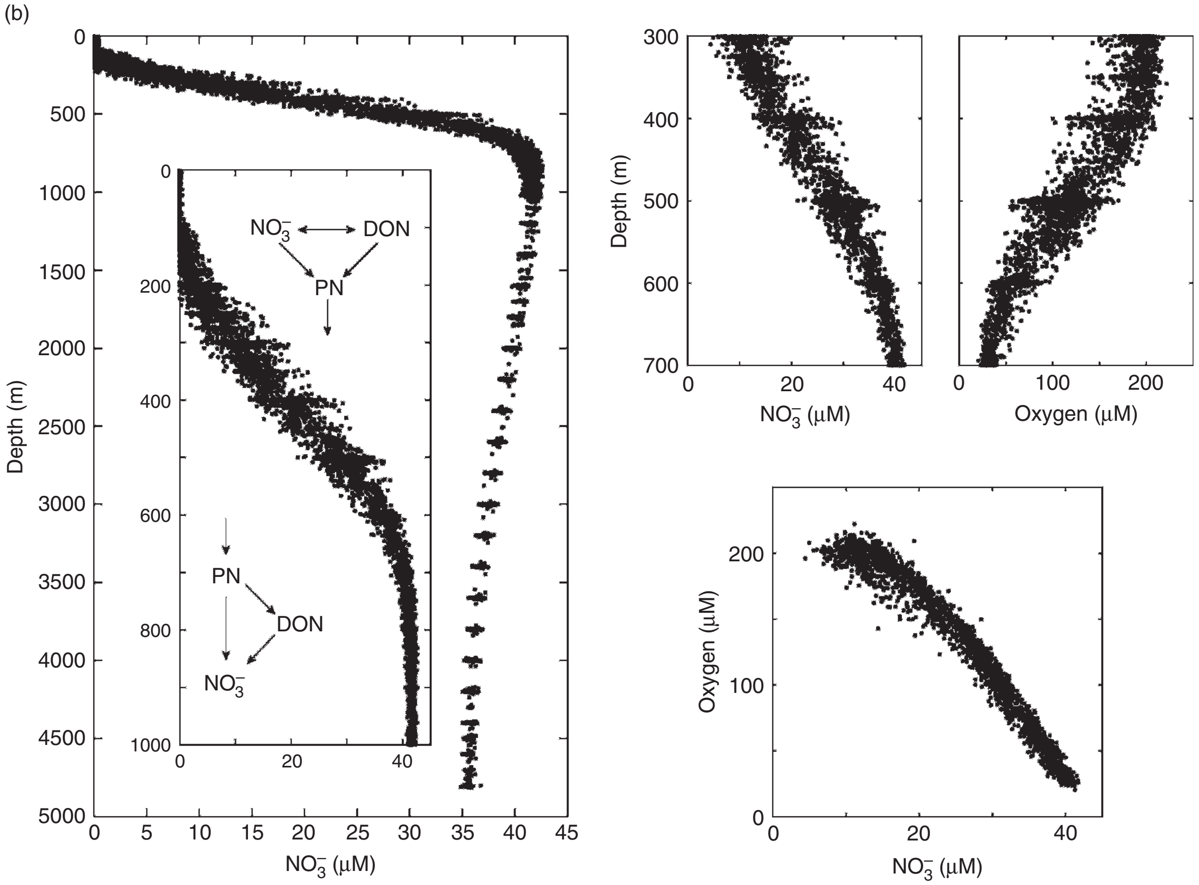

Figure 5 (**a**) Nitrate (NO$_3^-$) versus depth profiles for the North Atlantic (Bermuda Atlantic Time-series Study; BATS) and the North Pacific (Hawaii Ocean Time-series; HOT) showing significant interocean differences including a steeper nitracline (i.e., a larger change in NO$_3^-$ concentration per meter in the upper mesopelagic zone region) and higher deep water (>4000 m) NO$_3^-$ concentrations for HOT. These differences in NO$_3^-$ inventories and gradients, part of a systematic global pattern (see **Figure 6**), have significant implications for NO$_3^-$ fluxes into the euphotic zone. Data available at the HOT and BATS program websites (http://hahana.soest.hawaii.edu; http://www.bios.edu/). (b) Relationships between the vertical distributions of nitrate (NO$_3^-$) and dissolved oxygen (O$_2$) at Station ALOHA in the North Pacific Subtropical Gyre (NPSG). (Left) Graph of NO$_3^-$ (μmol l^{-1}) versus depth (m) showing the characteristic 'nutrient-like' distribution of NO$_3^-$ with regions of net NO$_3^-$ uptake and DON cycling and particulate nitrogen (PN) export near the surface, and net NO$_3^-$ remineralization at greater depths. The insert shows these main N-cycle processes, which are most intense in the upper 1000 m of the water column. (Right, top) NO$_3^-$ and O$_2$ concentration versus depth profiles of the 300–700 m region of the water column at Station ALOHA showing the effects of net remineralization of organic matter. (Right, bottom) A model 2 linear regression analysis of NO$_3^-$ versus O$_2$ suggests an average consumption of 80 μmol l^{-1} O$_2$ for each 1 μmol of NO$_3^-$ that is regenerated from particulate and DOM. Data available at the HOT program website (http://hahana.soest.hawaii.edu).

Pliocene nearly 10^7 years before present. The vertical water column at Station ALOHA can be partitioned into three major microbial habitats: euphotic zone, mesopelagic (twilight) zone, and aphotic zone (**Table 3** and **Figure 7**). The main determinant in this classification scheme is the presence or absence of light. The euphotic zone is the region where most of the solar energy captured by phototrophic marine microbes is sufficient to support photosynthetic activity. In the twilight zone (200–1500 m), light is present at very low photon fluxes, below which photosynthesis can occur, but at sufficiently high levels to affect the distributions of mesozooplankton and nekton and, perhaps, microbes as well. At depths greater than ~1500 m, light levels are less than 10^3 quanta cm^{-2} s^{-1}; the aphotic zone is, for all intents and purposes, dark.

Each of these major habitats is characterized by specific physical and chemical gradients, with distinct temporal scales of variability, providing unique challenges to the microorganisms that live there, and resulting in a vertical segregation of taxonomic structure and the ecological function of the resident microbial assemblages. A recent report of microbial community genomics at Station ALOHA, from the ocean's surface to the abyss, has revealed significant changes in metabolic potential, attachment and motility, gene mobility, and host–viral interactions.

The NPSG is characterized by warm (>24 °C) surface waters with relatively high light and relatively low

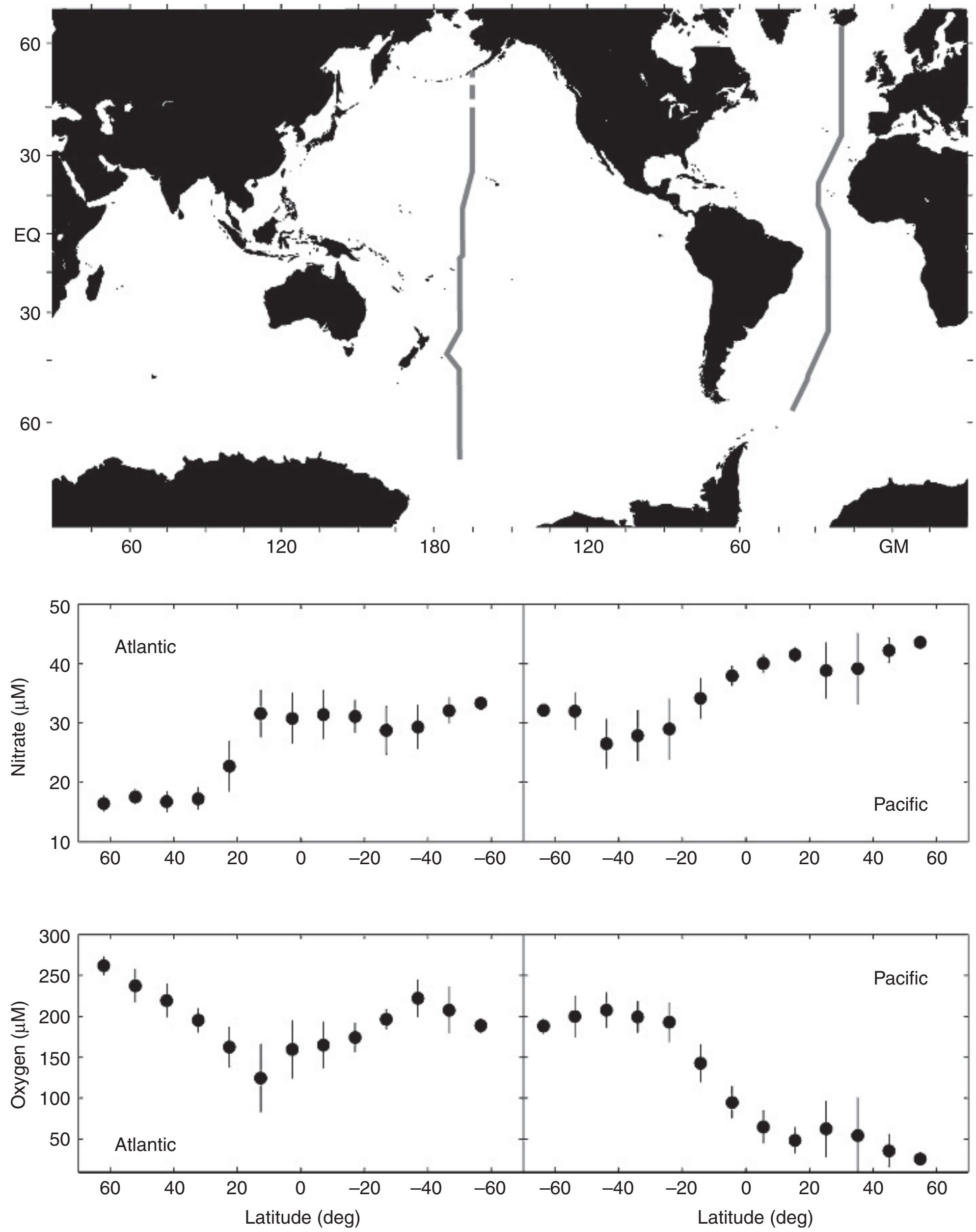

Figure 6 Map showing the locations of the World Ocean Circulation Experiment (WOCE) program transects A-16 (Atlantic) and P-15 (Pacific). Data from these cruises were obtained from http://woce.nodc.noaa.gov and averaged over the depth range of 500–1500 m, then combined into 10° latitude bins and plotted as mean nitrate and dissolved oxygen concentrations (±1 standard deviation). The resultant plot shows a systematic increase in nitrate concentrations at mid-water depths 'down' the Atlantic and 'up' the Pacific, and an opposite trend for oxygen. These spatial patterns are the result of the time-integrated aerobic decomposition of organic matter along known pathways of deep water circulation.

concentrations of inorganic nutrients and low microbial biomass (**Figure 8**). The euphotic zone has been described as a 'two-layer' habitat with an uppermost light-saturated, nutrient-limited layer (0–100 m) which supports high rates of primary productivity and respiration, and a lower (>100 m) light-limited, nutrient-sufficient layer. A region of elevated chl a, termed the Deep Chlorophyll Maximum Layer (DCML), defines the boundary between the two layers (**Figure 9**). The DCML in the NPSG results from photoadaptation (increase in chl a per cell) rather than enhanced phototrophic biomass; this can also be seen in the near-surface 'enrichment' of chl in winter when light fluxes are at their seasonal minimum (**Figure 9**).

Previously considered to be the oceanic analogue of a terrestrial desert, the NPSG is now recognized as a region of moderate primary productivity

Table 3 Conditions for microbial existence in the three major habitats at Station ALOHA in the North Pacific Subtropical Gyre

Habitat	*Depth range (m)*	*Conditions*
Euphotic zone (nutrient-limited)	0–200	• high solar energy • high DOM • low inorganic nutrients, trace elements, and organic growth factors
Mesopelagic (twilight) zone (transition)	200–1000	• low solar energy • decrease in reduced organic matter with depth • increase in organic nutrients and trace elements with depth
Abyssal zone (energy-limited)	>1000	• no solar energy • low DOM • high inorganic nutrients and trace elements

(150–200 g carbon m^{-2} year^{-1}), despite chronic nutrient limitation. Furthermore, based on data from the HOT program it appears that the rates of primary production have increased by nearly 50% between the period 1989 and 2006 due in part to enhanced nutrient delivery resulting from climate controls on habitat structure and function.

At Station ALOHA the light-supported inorganic carbon assimilation extends to 175 m, a depth that is equivalent to the 0.05% surface light level (~20 mmol quanta m^{-2} day^{-1}). Most of the light-driven inorganic carbon assimilation (>50%) occurs in the upper 0–50 m of the water column (**Figure 8**), a region of excess light energy (>6 mol quanta m^{-2} day^{-1}). In addition, chemoorganoheterotrophic microbial activities are also greatest in the upper 0–50 m. However, unlike photolithoautotrophic production (where light is required as the energy source and inorganic carbon is assimilated for growth), the metabolism of chemoorganoheterotrophs is not dependent on light energy so it continues, albeit at a reduced rate, well into the twilight zone and beyond. Recently, it has been observed that 'heterotrophic production' at Station ALOHA is enhanced by sunlight, suggesting the presence of microorganisms using light and both inorganic and organic substrate (photolithoheterotrophic) or light and organic substrates (photoorganoheterotrophic) to support their metabolism, or both. Several possible pathways for solar energy capture and carbon flux potentially exist in the euphotic zone at Station ALOHA, and we are just beginning to establish a

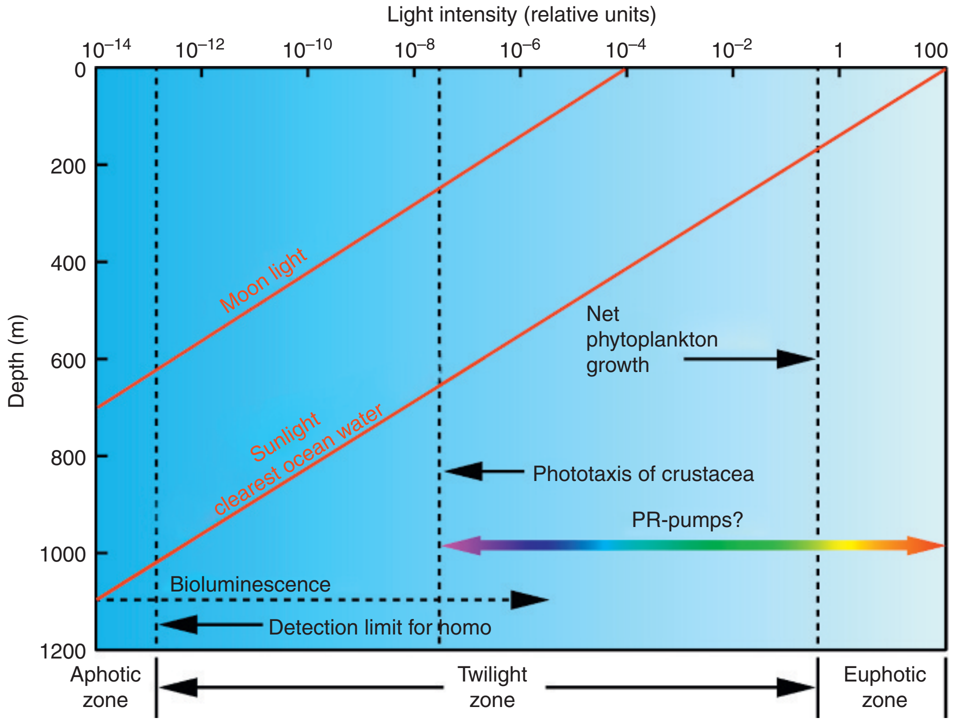

Figure 7 Schematic representation of the distribution of light in open ocean marine habitats. The *X*-axis displays light intensity (on a $\log_{10}$ scale in relative units) and the *Y*-axis is water depth. The euphotic zone where net photosynthesis can occur extends to a depth of ~150–200 m but sunlight can be detected by mesozooplankton (crustacean and fish) to depths of 800 m or more. The dark adapted human eye can detect even lower light fluxes. Proteorhodopsin proton pumps that have recently been detected in marine bacteria may also be able to use light but this is not yet confirmed. Moonlight, in contrast, is ~10^{-4} as bright as sunlight, but can also be detected by marine organisms and, perhaps, microbes. Bioluminescence, light production via cellular metabolism that can be found in nearly all marine taxa including microorgansisms, is found throughout the water column even in the 'aphotic' zone.

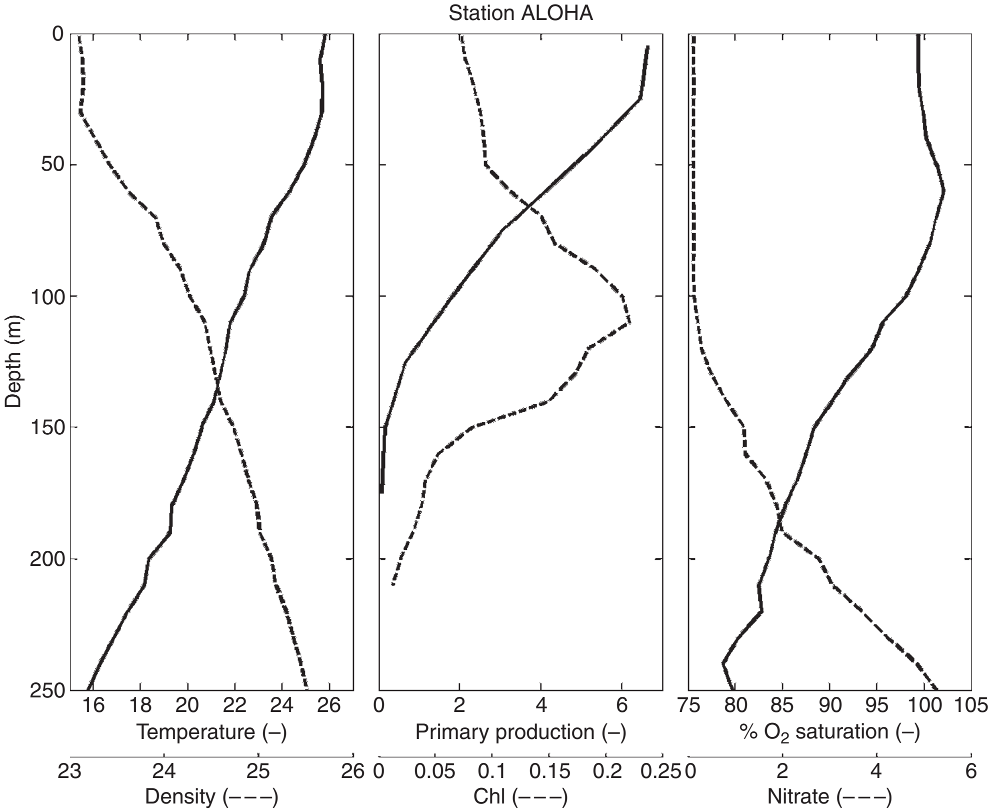

Figure 8 Typical patterns of the vertical distributions of selected physical and biological parameters at Station ALOHA in the NPSG. The base of the euphotic zone, defined here as the depth where primary production is equal to zero, is ~175 m. Units are: temperature (°C), density (shown as density anomaly; ((density g cm^{-3})$-$1.000) × 1000), primary production (mg C m^{-3} day^{-1}), chlorophyll (mg m^{-3}), nitrate (μM), and O$_2$ (% of air saturation). Compiled from the HOT program database (http://hahana.soest.hawaii.edu).

comprehensive understanding of these processes, their roles and controls, and the diversity of microbes supporting them in the pelagic ecosystem.

As described earlier, physical and chemical depth gradients in the water column affect the vertical distribution of microbial assemblages and their metabolic activities. Furthermore, at a macroscopic scale we can assess how each depth horizon is affected by different temporal patterns of variability, which, in turn, influence the microbial environment. For example, in the upper euphotic zone the variability in solar radiation due to cloud coverage and changes in day length associated with the seasonal solar cycle can affect the rates of photosynthesis. In this habitat, far removed from the upper nutricline (the depth at which nutrient concentrations start to increase), the dynamics of microbial processes will be controlled mainly by the rates of solar energy capture and recycling of nutrients through the food web. Furthermore, upper water column mixing rates also contribute significantly to the variability in the light environment. However, if the variability has a high frequency relative to the cell cycle, then microbes integrate the signal because the energy invested in acclimation may be greater than that gained by maximizing photosynthetic and photoprotective processes along the variability (light) gradient.

Between the base of the mixing layer and the top of the nutricline, the microbial assemblage resides in a well-stratified environment that is nevertheless still influenced by variability in light. Although mixing does not play a significant role in this habitat, unless a deep wind- or density-driven mixing event occurs, the vertical displacements of this stratified layer as the result of near-inertial period (~31 h at the latitude corresponding to Station ALOHA) oscillation forces may introduce strong day-to-day variability in the light availability and photosynthetic rates (**Figure 10**); these vertical motions can affect the short-term balance between photosynthesis and respiration. The variability in solar irradiance described above propagates into the lower euphotic zone, penetrating into the upper nutricline. But the apparent presence of excess nutrients relative to the bioavailable energy that can be derived through photosynthesis in this region indicates that light is the limiting factor supporting microbial activity. For this reason, day-to-day variations, as well as the seasonal cycle of solar irradiance in this layer may trigger successional patterns in the microbial

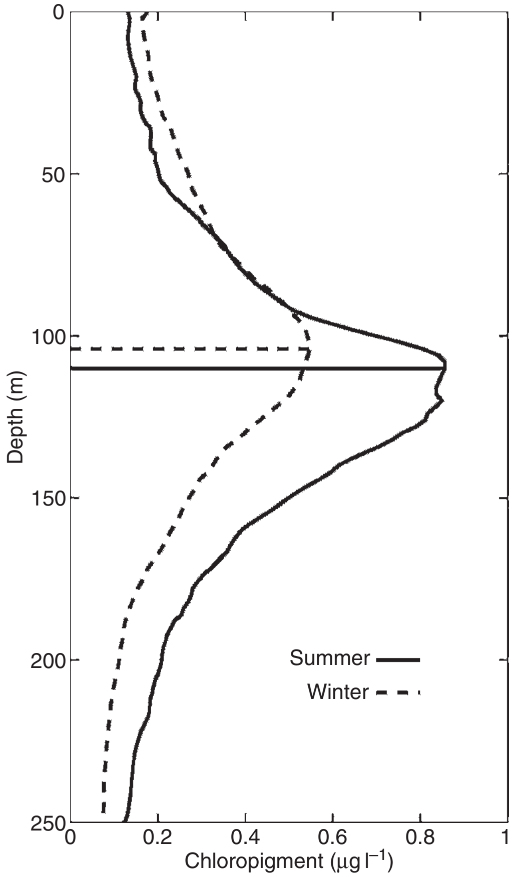

Figure 9 Vertical distributions of chloropigments (chlorophyll plus pheophytin) determined from *in vivo* fluorescence measurements and bottle calibrations. These graphs show average distributions at Station ALOHA for summer (June–Aug) versus winter (Dec–Feb) for 1999 showing and documenting changes in both total concentration of chloropigments at the surface and at the depth of the Deep Chlorophyll Maximum Layer (DCML). Both of these seasonal differences are caused primarily by changes in light intensity.

assemblage, and lead to pulses of organic matter export into the deeper regions of the ocean.

At Station ALOHA, as well as in most oceanic regions, the gravitational flux of particles formed in the euphotic zone represents the major source of energy that links surface processes to the deep sea. In addition, these sinking organic particles represent energy- and nutrient-enriched microhabitats that can support the growth of novel microbial assemblages. The remineralization of particles with depth follows an exponential decay pattern indicating that most of the organic matter in these particles is respired in the upper layer below the euphotic zone. If the quality and quantity of organic rain was constant, we would expect to observe stable layers of microbial diversity and activity with depth. However, the long-term records of particle flux to abyssal depths at Station ALOHA suggest that, during certain periods of the year, this flux increases significantly, representing potential inputs of organic matter into these deep layers driven by changes in upper water column microbial processes. Microscopic analysis of these organic matter pulses at Station ALOHA reveal that their composition is dominated by diatoms. These photolithoautotrophic microbes produce an external siliceous skeleton that can act as strong ballast when the cells become senescent.

Several mesoscale physical processes have been observed that can modify the upper water column habitat at Station ALOHA, triggering an increase in the relative abundance of diatoms in surface waters and subsequent cascade of ecological processes. The passage of mesoscale features, such as eddies and Rossby waves, can shift the depth of nutrient-rich water relative to the euphotic zone, leading to a possible influx of nutrients into the well-lit zone that can last from days to weeks. This sustained nutrient entrainment can alter the microbial size spectrum, in favor of rapidly growing, large phytoplankton cells (usually diatoms), resulting in a bloom. In addition, eddies can trap local water masses and transport microbial assemblages for long distances.

A second mechanism triggering changes in the microbial community appears to occur during summer months at Station ALOHA, when the upper water column is warm and strongly stratified. Under these conditions, N_2-fixing cyanobacteria, sometimes living in symbiosis with diatoms, aggregate in surface waters and provide an abundant supply of reduced nitrogen and organic matter to the microbial community. Although it is still not clear what triggers these summer blooms, *in situ* observations suggest that they significantly alter the structure and metabolic activity of the microbial assemblage.

Finally, the mixing layer can periodically penetrate to a depth where it erodes the upper nutricline and delivers nutrients to the surface waters, while mixing surface-dwelling microbes into the upper nutricline. This deepening of the mixing/mixed layer can be driven by sudden events such as the development of a severe storm or the cooling of surface waters by the passage of a cold air mass. And, although each of these three mechanisms can lead to the entrainment of nutrients into the euphotic zone, they generate different microbial responses and interaction. For example, while the first two mechanisms do not involve a change in stratification, the third mixes the water column temporarily erasing the physical, chemical, and biological gradients that had existed before the event. Furthermore, while the passage of eddies and Rossby waves introduce nutrients into the base of the euphotic zone, affecting primarily the microbial populations inhabiting the upper nutricline, summer blooms have their strongest effect in the microbial assemblages residing in the upper few meters of the water column. Nevertheless, all these mechanisms appear to generate pulses of particulate organic matter rain that enhance the availability of

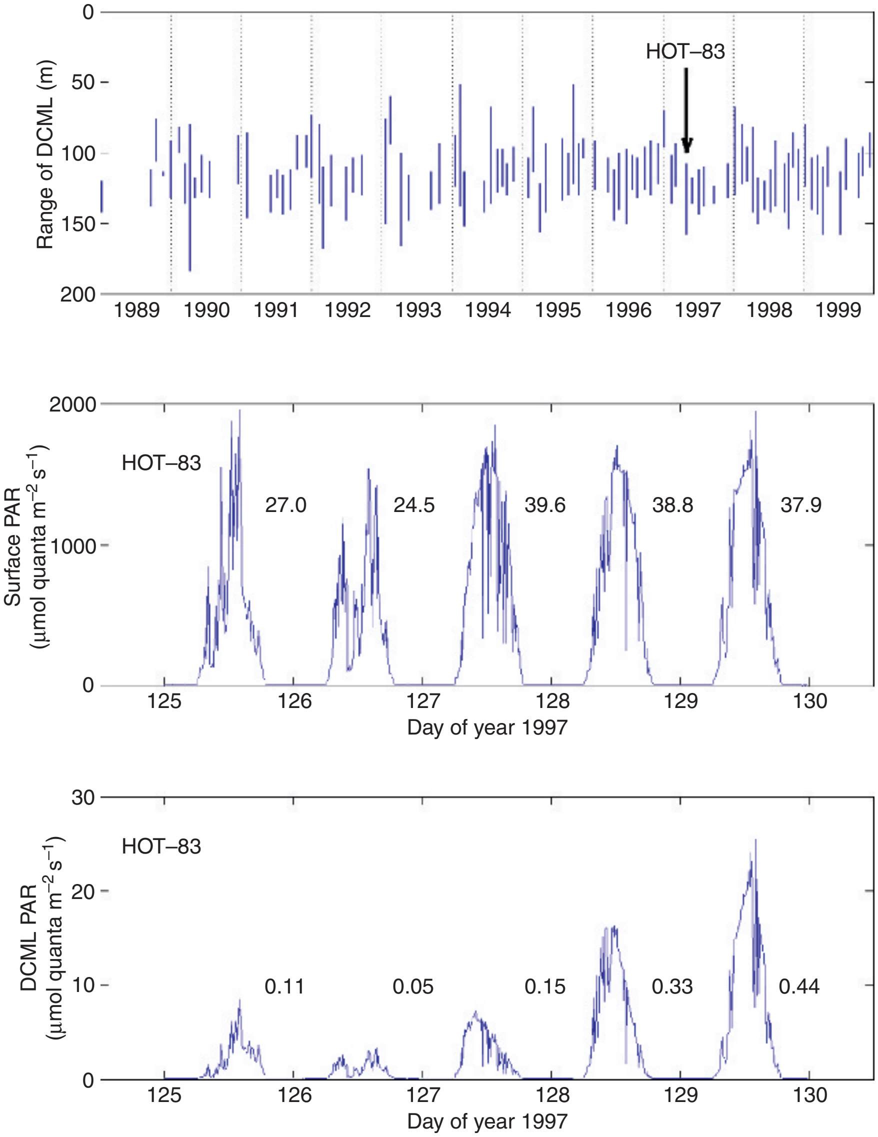

Figure 10 Effect of isopycnal vertical displacements in accounting for day-to-day variability of Photosynthetically Available Radiation (PAR) at the DCML at Station ALOHA: (Top) Observed minimum and maximum depth range distribution of the DCML for each HOT cruise based on continuous fluorescence trace profiles obtained from 12 CTD casts deployed over a 36-h sampling period. (Center) Surface PAR measured at the HALE ALOHA mooring location during HOT-83 (5–9 May 1997). (Bottom) Estimated PAR at the DCML based on the vertical displacement of the DCML, surface PAR, and assuming $k_{PAR} = 0.04\,m^{-1}$. Daily integrated PAR values (in mol quanta m^{-2} day^{-1}) are displayed next to each light cycle in (Center) and (Bottom). These day-to-day variations in light caused by inertial period oscillations of the DCML and variations in surface PAR due to clouds are certain to have significant effects on rates of *in situ* photosynthesis. Reproduced from Karl DM, Bidigare RR, and Letelier RM (2002) Sustained and aperiodic variability in organic matter production and phototrophic microbial community structure in the North Pacific Subtropical Gyre. In: Williams PJ, le B, Thomas DR, and Reynolds CS (eds.) *Phytoplankton Productivity and Carbon Assimilation in Marine and Freshwater Ecosystems*, pp. 222–264. London: Blackwell Publishers.

ephemeral microenvironments, fuel the deeper microbial layers, and carry microbes to depth.

In addition to mesoscale events and seasonal cycles that seem to support small transient changes in the microbial community structure and function, variability at longer time scales (interannual to decadal) may shift the taxonomic structure of the microbial community. For example, there is evidence suggesting that a significant shift in the dominance of phototrophic taxa may have taken place in the NPSG as a result of changes in ocean circulation and wind forcing during the 1970s. More recently, changes in the stability of the upper water column since the 1997–98 El Niño event may have also triggered long-term changes in the phototrophic community structure.

Ultimately, these long-term habitat changes are the result of processes taking place over a broad range of scales propagating into the habitat experienced by a microbe. In this context, the advent of novel molecular tools such as metagenomic, proteomic, and transcriptomic analyses has provided an unprecedented opportunity to infer the diversity and biogeochemical relevance of microhabitats via the characterization of the genes being expressed in the environment. These new tools may help us better explore how physical and biological processes, by affecting the spatial and temporal distribution of these habitats, shape the microbial diversity and metabolism in the sea. However, understanding how microbial assemblages in different oceanic habitats may evolve over time in response to climate change will require not only a characterization of the microbes' response to physical and chemical changes, but also the development of an understanding of how interactions among microbes contribute to the plasticity and resilience of the microbial ecosystem in the marine environment.

Summary and Prospectus

All marine habitats support diverse microbial assemblages that interact through a variety of metabolic and ecological processes. The characteristics and dynamics of marine habitats determine the composition, structure, and function of their microbial inhabitants. Many microbial habitats (i.e., microhabitats) are cryptic, ephemeral, and difficult to observe and sample; the spatial and temporal domains of these environments are poorly resolved at present. The changing ocean will lead to different and, probably, novel marine habitats that will select for new microbial assemblages. Future ecological research should focus on the relationships among climate, habitat, microbes, and their individual and collective metabolic function. These comprehensive studies demand coordinated, transdisciplinary field programs that fully integrate physical and chemical oceanography with theoretical ecology into the wonderful world of marine microbes.

Acknowledgments

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Metabolism, Central (Intermediary)

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Defining Statement
Introduction
Central Metabolic Pathways
Gluconeogenesis
Utilization of Polysaccharides, Oligosaccharides, and Monosaccharides
Precursor Metabolites for Biosynthesis/Anabolism
Further Reading

Glossary

aerobic The presence of oxygen.

anaerobic The absence of oxygen.

anapleurotic reaction A metabolic reaction that functions to replenish one or more intermediates of a pathway.

carbohydrate Organic molecules composed of carbon–hydrogen–oxygen typically in a 1:2:1 ratio; a sugar or a derivative thereof.

carbon-energy source An organic molecule that can be metabolized by the cell to generate energy and to produce carbon backbone intermediates/precursors for anabolic processes.

catabolism The branch of metabolism in which complex larger molecules are broken down into less complex smaller molecules with the concomitant release of energy.

(chemo)heterotrophic Refers to the requirement of some organisms to obtain their carbon from organic molecules.

electron transport phosphorylation The major mechanism of ATP production during respiration; results from the proton motive force generated by the passage of electrons through the electron transport system and concurrent extrusion of protons from the cytoplasm, which provides the energy for the production of ATP from ADP plus P_i.

fermentation The process of generating energy from the oxidation of organic molecules that serve as both electron donors and acceptors in the process.

gluconeogenesis The synthesis of hexose sugar intermediates of central metabolic pathways for the purpose of providing biosynthetic precursors during growth on carbon sources other than hexose sugars.

glycolysis The breakdown or catabolism of glucose (sugars); sometimes used when referring to the Embden–Meyerhof–Parnas glycolytic pathway but used here to refer to glycolytic pathways in general.

oxidation In general terms, it refers to the loss of electrons by a molecule, ion, or atom.

reduction In general terms, it refers to the gain of electrons by a molecule, ion, or atom.

respiration The production of ATP from the catabolism of organic molecules in which the terminal electron acceptor is oxygen (or in some cases another exogenous electron acceptor such as nitrate).

substrate-level phosphorylation ATP production that results from the direct transfer of a phosphate group to ADP from an organic intermediate possessing a high-energy phosphate bond.

Abbreviations

ADP-HK	ADP-dependent hexokinase
ADP-PFK	ADP-dependent PFK
AMP	adenosine monophosphate
ATP-PFK	ATP-dependent PFK
1,3-BPG	1,3-bisphosphoglycerate
DHAP	dihydroxyacetone phosphate
KDPG	2-keto-3-deoxy-6-phosphogluconate
ED	Entner–Doudoroff pathway
EMP	Embden–Meyerhof–Parnas pathway
FBA	fructose bisphosphate aldolase
FBP	fructose-1,6-bisphosphate
FDH	formate dehydrogenase
FHL	formate-hydrogen lyase
GAP	glyceraldehyde-3-phosphate
GAPDH	glyceraldeyde-3-phosphate dehydrogenase
GAPOR	glyceraldehyde-3-phosphate ferridoxin oxidoreductase
HK	hexokinase
ICL	isocitrate lyase
KDG	2-keto-3-deoxygluconate
LALDH	lactaldehyde dehydrogenase
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MG	methylglyoxal

OAA	oxaloacetate	**PPK**	pentose phosphoketolase pathway
PDH	pyruvate dehydrogenase	**PPP**	pentose phosphate pathway
PEP	phosphoenolpyruvate	**PP-PFK**	pyrophosphate (PP_i)-dependent PFK
PFK	phosphofructokinase	**PRPP**	5-phospho-D-ribosyl-1-pyrophosphate
PFL	pyruvate–formate lyase	**PYK**	pyruvate kinase
PG	phosphoglycerate	**SDH**	succinate dehydrogenase
PGI	phosphoglucose isomerase	**SH-7P**	sedoheptulose-7-phosphate
PGK	phosphoglycerokinase	**TCA**	tricarboxylic acid cycle
PGM	phosphoglycerate mutase	**TPP**	thiamine pyrophosphate
PKP	phosphoketolase pathway		
pmf	proton motive force		
PP_i	pyrophosphate		

Defining Statement

Central metabolism provides mechanisms for the generation of energy from carbon-energy source catabolism and the production of biosynthetic precursors and intermediates for the anabolic pathways essential to maintain cell structure and function. Here, the central metabolic pathways are described and discussed in terms of the remarkable diversity of the microbial world.

Introduction

The beauty of the microbial world lies in the diversity and complexity of environments where microbes are found. Key to growth and survival of (chemo)heterotrophic microbes is the ability to utilize diverse collections of organic compounds as carbon and energy sources. These can range from carbohydrates (e.g., glucose the preferred carbon-energy source of most microbes), fatty acids, amino acids to nucleic acids and other compounds. Regardless of the myriad of organic substrates utilized by heterotrophic microbes, all are metabolized initially through substrate-specific pathways that feed into a common set of pathways collectively referred to as central (intermediary) metabolism. The functions of central metabolic pathways are to generate the majority of energy (typically in the form of high-energy phosphodiester bonds of adenosine triphosphate or ATP) and oxidation–reduction (O–R) cofactor molecules, nicotinamide adenine dinucleotide (NADH), and NAD phosphate (NADPH). Substrate-specific or 'feeder' pathways may also generate energy and O–R cofactors for the cell. In addition to their roles in generating the bulk of the energy and reducing power for the cell, various pathways of central metabolism produce the essential precursor metabolites (e.g., fructose-6-phosphate, ribose-5-phosphate, phosphoenolpyruvate (PEP), pyruvate, and α-ketoglutarate) necessary for *de novo* biosynthetic (i.e., anabolic) pathways of cellular metabolism (**Figure 1**).

Central Metabolic Pathways

Although glucose is the most preferred carbon-energy source used by most microbes, other carbohydrate/sugar and noncarbohydrate substrates can also serve as carbon-energy sources depending on the microbe. Typically, these alternative substrates are converted into intermediates that feed into one of the central metabolic pathways involved in glucose metabolism. Central metabolic pathways common to most microbes include the Embden–Meyerhof–Parnas (EMP) pathway and the pentose phosphate pathway (PPP). Additional pathways of carbon-energy source metabolism, for example, phosphoketolase pathway (PKP), Entner–Doudoroff (ED) pathway, and tricarboxylic acid (TCA) cycle, are present in different microbes and function under different conditions.

Pathways of Glucose Catabolism or Glycolysis

Embden–Meyerhof–Parnas pathway

The Embden–Meyerhof–Parnas (EMP) pathway is more commonly referred to as glycolysis (**Figure 2**). The degradation of glucose to two pyruvates (along with the generation of a net two $NADH/H^+$ and two ATP

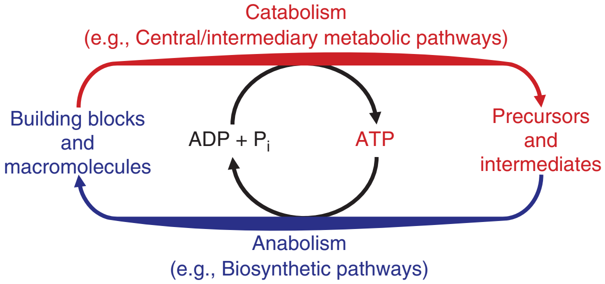
Catabolism
(e.g., Central/intermediary metabolic pathways)
Building blocks and macromolecules
ADP + P_i
ATP
Precursors and intermediates
Anabolism
(e.g., Biosynthetic pathways)

Figure 1 Schematic representation of the relationship between central metabolic pathways and the generation of ATP and the biosynthesis of building block molecules (e.g., amino acids, nucleotides) and macromolecules (e.g., proteins, DNA, RNA).

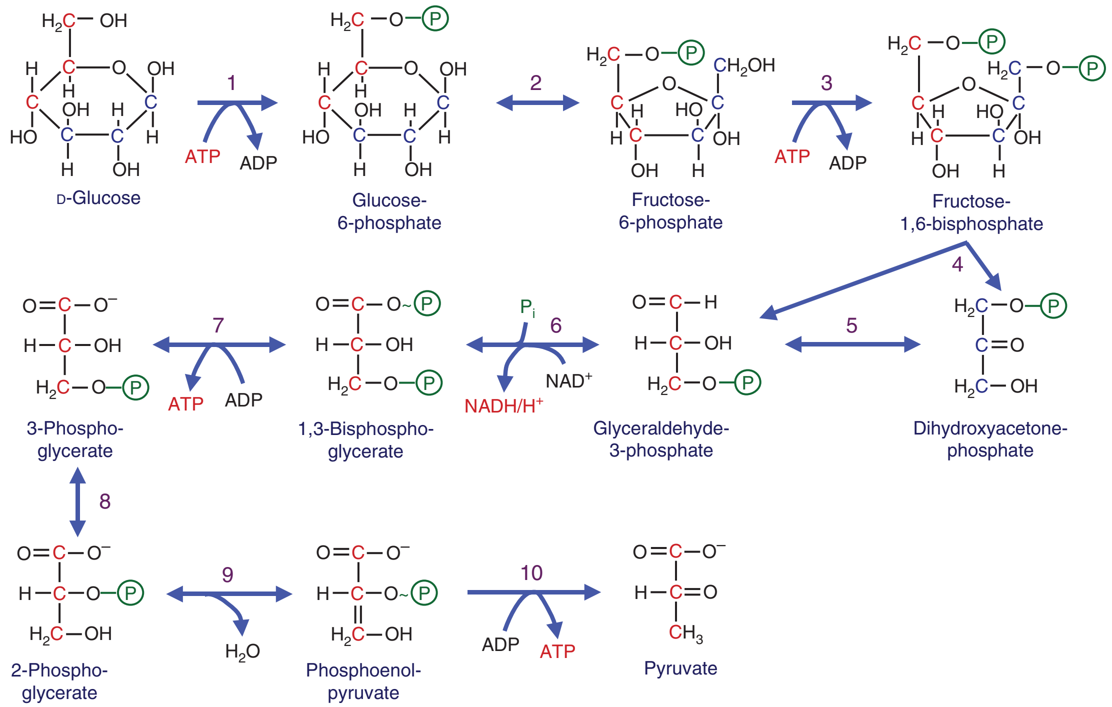

Figure 2 Embden–Meyerhof–Parnas (EMP) pathway. Enzymes catalyzing each step are as follows: (1) (ATP-dependent) hexokinase (HK; aka, glucokinase), (2) phosphoglucoisomerase (PGI), (3) (ATP-dependent) phosphofructokinase (PFK), (4) fructose bisphosphate aldolase (FBA), (5) triose phosphate isomerase, (6) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (7) phosphoglycerokinase (PGK), (8) phosphoglycerate mutase (PGM), (9) (phosphoglycerate) enolase, and (10) pyruvate kinase (PYK).

molecules) is essentially the same in bacteria and eukaryotic microbes that employ the EMP pathway. The EMP pathway is the major glucose catabolic pathway in many bacteria (e.g., *Bacillus subtilis*, *Lactobacillus* species, *Sarcina lutea*, *Streptomyces griseus*, and the enterobacteria) and all yeasts (e.g., *Saccharomyces cerevisiae*). Pyruvate is the point of divergence in that it can be further catabolized through several different biochemical reactions to a variety of products, for example, acids, alcohols, aldehydes, gases, or through the TCA cycle depending on the microbe and the environmental conditions, for example, aerobic versus anaerobic conditions (discussed further below).

In the first step of the EMP pathway, D-glucose is phosphorylated by the enzyme hexokinase (HK; aka, glucokinase) to D-glucose-6-phosphate (Glc-6P) using ATP. The cleavage of the high-energy γ-phosphoric acid anhydride bond of ATP coupled to the transfer of the phosphate group and formation of the lower-energy phospho-ester bond in Glc-6P drives the reaction. The phosphorylation of sugars is essential for them to be further metabolized; this is commonly carried out by cytoplasmic enzymes such as HK that utilize ATP or during uptake into the cytoplasm by various transport systems using either ATP or PEP (i.e., PEP:sugar phosphotransferase system) depending on the sugar and microorganism. The negatively charged sugar phosphate is then prevented from leaking out of the cytoplasm and the cell.

In the next step, the enzyme phosphoglucoisomerase (encoded by the *pgi* gene in *Escherichia coli* and other bacteria) mediates the isomerization of Glc-6P to fructose-6-phosphate (Fru-6P). This rearrangement produces a more compact and lower entropy molecule. The third step of the pathway involves the phosphorylation of Fru-6P to fructose-1,6-bisphosphate (FBP) mediated by the enzyme phosphofructokinase (PFK; encoded by the *pfkA* gene in *E. coli* and other bacteria) using ATP as discussed above. This creates a compact molecule with two negatively charged phosphate groups very close together producing some intramolecular instability. The first three reactions of the pathway function to prepare the six-carbon (C_6) glucose molecule for separation into two three-carbon (C_3) triose phosphate molecules at the expense of the hydrolysis of two ATP molecules.

In the fourth step of the pathway, the FBP formed is cleaved to Glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by the enzyme fructose bisphosphate aldolase (FBA; encoded by the *fbaA* gene in *E. coli* and other bacteria). The two triose phosphates, GAP and DHAP, can be interconverted by the enzyme triose phosphate isomerase. This allows both triose phosphate products to be further metabolized through the triose phosphate portion of the EMP pathway. GAP, during the next step, is oxidized and an inorganic phosphate (P_i) is added to the molecule to form

1,3-bisphosphoglycerate (1,3-BPG). The enzyme glyceraldeyde-3-phosphate dehydrogenase (GAPDH; encoded by the *gap* gene in *E. coli* and other bacteria) utilizes the high-energy potential of oxidation to add a P_i group to the molecule. In this process, GAP oxidation and phosphorylation is coupled to the reduction of NAD^+ to $NADH/H^+$. 1,3-BPG is a mixed acid anhydride possessing a high-energy anhydride phosphate bond. The generation of this intermediate sets up the generation ATP during the final reactions of this pathway.

In the next step of this glycolytic pathway, phosphoglycerokinase (PGK; encoded by the *pgk* gene in *E. coli* and other bacteria) takes 1,3-BPG plus adenosine diphosphate (ADP) and forms 3-phosphoglycerate (3-PG) and ATP. The remaining phosphate ester bond in 3-PG has relatively low free energy of hydrolysis. The subsequent two steps in the pathway function to form a high-energy bond that can be linked to the generation of a second ATP during the final step in the EMP pathway. The 3-PG formed in step seven undergoes intramolecular rearrangement, with the phosphate group being moved from the third carbon to the second carbon to form 2-phosphoglycerate (2-PG), catalyzed by the enzyme phosphoglycerate mutase (PGM; encoded by the *pgm* gene in *E. coli* and other bacteria). In the following step, a water molecule is removed from 2-PG to form a high-energy enolphosphate bond in PEP; this reaction is catalyzed by the enzyme (PG) enolase (encoded by the *eno* gene in *E. coli* and other bacteria). In the final step of the EMP pathway, pyruvate kinase (PYK; encoded by the *pykA* and *pykF* gene in *E. coli* and other bacteria) transfers the high-energy phosphate group from PEP to ADP forming pyruvate and ATP. The formations of ATP in the phosphoglycerokinase- and pyruvate kinase-mediated steps are examples of substrate level phosphorylation.

Thus, the catabolism of one six-carbon glucose molecule to two three-carbon pyruvate molecules yields a net of two $NADH/H^+$ and two ATP molecules. The EMP pathway accounts for the majority of carbon flux within those microbes possessing the pathway. For some bacteria known as homofermentative lactic acid bacteria (e.g., species of *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus*, as well as *Lactobacillus casei*, *Lactobacillus pentosus*, and *Lactobacillus plantarum*) the EMP pathway is the only glycolytic pathway and, therefore, substrate level phosphorylation is the sole mode of ATP production in these bacteria.

The genes for the enzymes composing the EMP pathway are believed to be constitutively expressed; however, in the facultative anaerobe *E. coli* and in many other bacteria the *gap* (glyceraldehydes-3-phosphate dehydrogenase) and *pgk* (phosphoglucokinase) genes are both upregulated by glucose in a catabolite-control protein A (CcpA)-dependent manner. Several of the enzymes themselves exhibit either positive or negative feedback control by pathway intermediates and/or adenosine monophosphate (AMP) or ADP. For example, PFK is positively regulated by ADP and negatively regulated by PEP while pyruvate kinases are positively regulated by FBP and/or AMP. PFK is the rate-limiting enzyme of the EMP pathway.

Pentose phosphate pathway

Although the majority of carbon-source catabolism occurs through the EMP pathway, as much as one-fifth of the glucose/hexose molecules can be catabolized through the PPP (aka, hexose monophosphate pathway/shunt) in microbes possessing both pathways. Mutants blocked in the EMP pathway enzyme PFK are able to grow on glucose-6-phosphate indicating that this pathway can function as a major glucose catabolic pathway when needed. For *Gluconobacter* (*Acetobacter*) *suboxydans*, a modified PPP appears to be the only glucose catabolic pathway. For others (e.g., *Neisseria perflava* and *Neisseria sicca*) the PPP is a major route for glucose dissimilation. Generally, the primary functions of the PPP is (1) production of NADPH needed for reductive steps in numerous biosynthetic pathways (e.g., α-ketoglutarate conversion to glutamate and fatty acid biosynthesis), and (2) production of intermediates (e.g., pentose (5-C), tetrose (4-C), and heptose (7-C) phosphates) needed for biosynthesis of nucleotides, amino acids, and lipopolysaccharide (LPS) as well as other precursor molecules.

The first three steps of the PPP are referred to as the oxidative branch since the reduction of 2 $NADP^+$ to 2 $NADPH/H^+$ molecules is coupled to the oxidation of glucose-6-phosphate (Glc-6P), formed from the phosphorylation of D-glucose by (ATP-dependent) hexokinase (ATP-HK), to ribulose-5-phosphate plus CO_2 (**Figure 3**(**a**)). In the first step, Glc-6P is converted to 6-phosphate-gluconolactone by glucose-6-phosphate dehydrogenase (Glc-6PDH; encoded by the *zwf* gene in *E. coli* and other bacteria, *zwf* comes from zwischenferment); this oxidation step is coupled to the reduction of a $NADP^+$ to form the first $NADPH/H^+$ molecule. 6-Phosphate-gluconolactone is then converted to 6-phosphogluconate by 6-phosphogluconolactonase (encoded by the *pgl* gene in *E. coli* and other bacteria) using one H_2O molecule. The conversion of 6-phosphate-gluconolactone to 6-phosphogluconate can also occur nonenzymatically at a significant level. In the next step, 6-phosphogluconate is oxidatively decarboxylated to form ribulose-5-phosphate plus CO_2 by 6-phosphogluconate dehydrogenase (encoded by the *gnd* gene in *E. coli* and other bacteria). During this step, a $NADP^+$ is reduced to form a second $NADPH/H^+$ molecule.

The remaining steps of the PPP represent the nonoxidative branch of the pathway (**Figure 3**(**b**)). The ribulose-5-phosphate that is formed is converted to either ribose-5-phosphate by ribose-5-phosphate isomerase

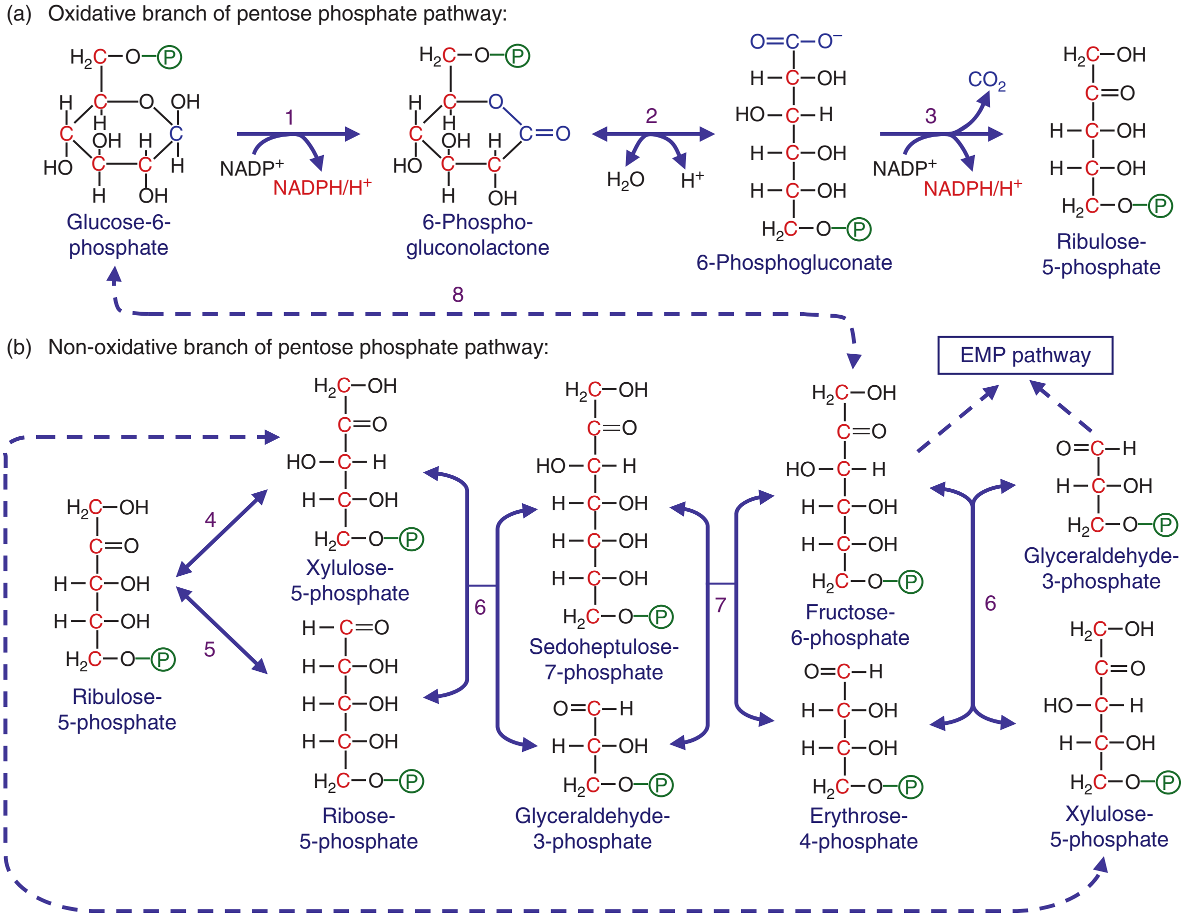
(a) Oxidative branch of pentose phosphate pathway:
Glucose-6-phosphate
1
NADP+
NADPH/H+
6-Phosphogluconolactone
2
H2O
H+
6-Phosphogluconate
3
CO2
NADP+
NADPH/H+
Ribulose-5-phosphate
8
(b) Non-oxidative branch of pentose phosphate pathway:
EMP pathway
Ribulose-5-phosphate
4
5
Xylulose-5-phosphate
Ribose-5-phosphate
6
Sedoheptulose-7-phosphate
Glyceraldehyde-3-phosphate
7
Fructose-6-phosphate
Erythrose-4-phosphate
6
Glyceraldehyde-3-phosphate
Xylulose-5-phosphate

Figure 3 Pentose phosphate pathway (PPP; aka, hexose monophosphate pathway). Enzymes catalyzing each step are as follows: (1) glucose-6-phosphate dehydrogenase (Glc-6PDH), (2) 6-phosphogluconolactonase, (3) 6-phosphogluconate dehydrogenase, (4) ribulose-5-phosphate epimerase, (5) ribose-5-phosphate isomerase, (6) transketolase, (7) transaldolase, and (8) phosphoglucoisomerase.

(encoded by the *rpiA* genes in *E. coli* and other bacteria) or xylulose-5-phosphate by ribulose-5-phosphate epimerase (encoded by the *rpe* gene in *E. coli* and other bacteria). These three pentose phosphates are maintained in equilibrium by these two enzyme activities. *E. coli* and some other bacteria also possess a second gene, *rpiB*, encoding a ribose-5-phosphate isomerase activity; however, based on genetic analysis the *rpiA* enzyme is required for conversion of ribulose-5-phosphate to ribose-5-phosphate in the PPP while the *rpiB* enzyme only allows for growth on ribose as a carbon-energy source unless it is overexpressed. Both the ribulose-5-phosphate epimerase and ribose-5-phosphate isomerase activities must be functional for the continuation of the pathway.

In the next few steps, xylulose-5-phosphate and ribose-5-phosphate are converted to sedoheptulose-7-phosphate (SH-7P) and glyceraldehyde-3-P (GAP) by the enzyme transketolase (encoded by the *tkt* gene in *E. coli* and other bacteria). The enzyme transaldolase (encoded by the *tal* gene in *E. coli* and other bacteria) mediates the conversion of SH-7P and GAP to fructose-6-phosphate (Fru-6P) and erythrose-4-phosphate (Ery-4P). Transketolase also catalyzes the conversion of Ery-4P and xylulose-6-phosphate to Fru-6P and GAP. This branch of the PPP supplies key intermediates, Fru-6P and GAP, that can feed into the EMP pathway providing a mechanism for growth on pentoses (e.g., xylulose, xylose, ribose, or ribulose) and gluconate as carbon-energy sources. In addition, this pathway provides the intermediates SH-7P and Ery-4P required for biosynthesis of LPS and aromatic amino acids (e.g., tyrosine, phenylalanine, and tryptophan), as well as vitamins/cofactors (e.g., folates, ubiquinone, menaquinone), respectively.

The Fru-6P formed in the transaldolase and subsequent transketolase reactions can be converted to Glc-6P by the EMP enzyme phosphoglucoisomerase producing a potential cyclic nature to this pathway. Several turns of this cycle could result in the complete oxidation of Glc-6P (+12 $NADP^+$) to $6CO_2$ (+12$NADPH/H^+$). However, such a cycle does not appear to function at a significant level under normal conditions.

Entner–Doudoroff pathway

The Entner–Doudoroff (ED) pathway is present in a number of bacteria where it can be a major pathway of glucose catabolism under aerobic conditions. The ED pathway (**Figure 4**) represents an offshoot of the oxidative branch of the PPP. Glucose-6-phosphate, formed from the phosphorylation of D-glucose by ATP-dependent hexokinase, is converted to 6-phosphogluconate by the subsequent actions of Glc-6P dehydrogenase (Glc-6PDH) and 6-phosphogluconolactonase producing one NADPH/H^+. 6-Phosphogluconate is then converted to 2-keto-3-deoxy-6-phosphogluconate (KDPG) through the removal of a water molecule by the enzyme 6-phosphogluconate dehydratase. KDPG is then split by the enzyme KDPG aldolase into pyruvate and GAP. GAP can then be converted to pyruvate through the triose phosphate portion of the EMP pathway producing two ATP and one NADH/H^+. Thus, a single glucose molecule catabolized through the ED pathway can be degraded to two pyruvates yielding a net one ATP plus one NADPH/H^+ and one NADH/H^+ depending on the microbe. As a result, the ED pathway yields half the net amount of energy in the form of ATP from the catabolism of a single glucose molecule to two pyruvates compared with the EMP pathway.

Although the ED pathway is most prevalent among strictly aerobic Gram-negative bacteria, including species of *Pseudomonas* (e.g., *Ps. aeruginosa*), *Agrobacterium* (e.g., *A. tumefaciens*), *Azotobacter* (e.g., *A. vinelandii*), *Xanthomonas*, *Arthrobacter*, *Caulobacter*, and *Neisseria* (e.g., *N. gonorrhoeae* and *N. meningitides*), it is also present in many Gram-negative facultative anaerobes such as *E. coli* and *Vibrio* spp. (e.g., *V. cholerae*) as well as the nitrogen-fixing *Sinorhizobium* (e.g., *S. meliloti*), photoheterotrophic *Rhodobacter* (e.g., *Rh. sphaeroides*), the nitrogen-oxidizing *Paracoccus* (e.g., *P. versutus*), and the cyanobacteria. Aerobic Gram-negative bacteria can live with the relatively low energy yield of the ED pathway because they obtain the majority of their energy through oxidative phosphorylation mechanisms. In these bacteria, the ED enzymes are typically inducible rather than constitutive since intermediates of the TCA cycle (e.g., citrate, succinate) rather than sugars are the preferred C-energy sources.

In *E. coli*, a gluconate permease and the genes (*edd–eda* operon) encoding the ED pathway enzyme 6-phosphogluconate dehydratase (*edd* for ED dehydratase) and KDPG aldolase (*eda* for ED aldolase) are induced by growth on gluconate. The *edd–eda* genes are induced from a GntR-regulated gluconate-responsive promoter, P_1, located upstream of *edd*. Interestingly, the *edd* gene is effectively not expressed in the presence of glucose while *eda* exhibits higher basal level expression from other promoters (P_2 or P_4) regardless of the C-energy source being utilized. The *eda* gene is also induced by glucuronate and galacturonate from the KdgR-regulated P_2 promoter. This likely plays a key role in growth of *E. coli* within

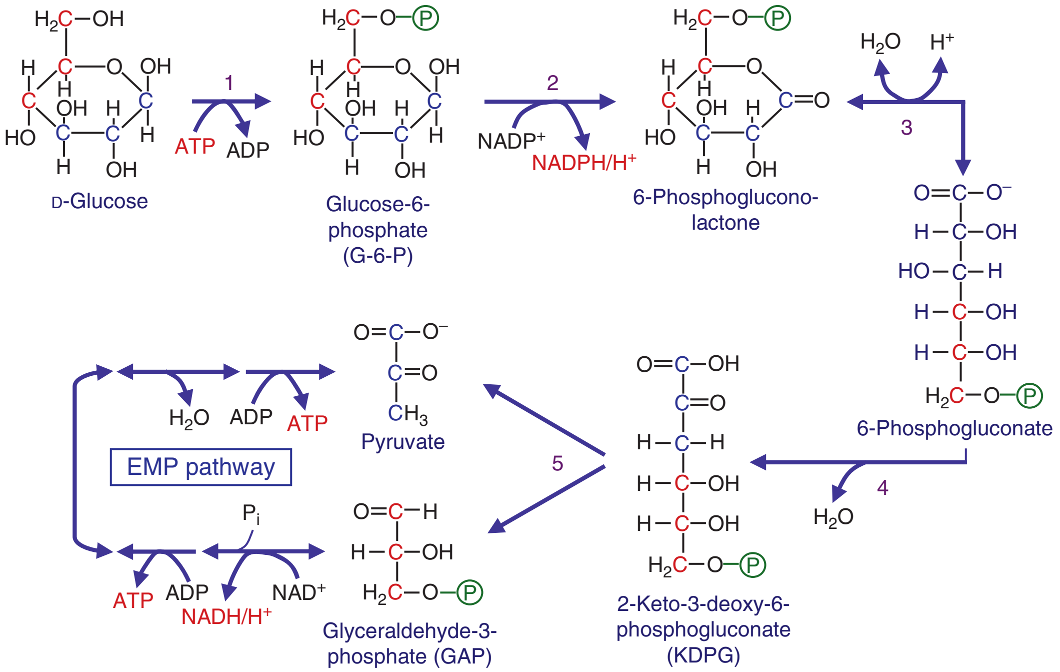

Figure 4 Entner–Doudoroff (ED) pathway. Enzymes catalyzing each step are as follows: (1) (ATP-dependent) hexokinase (aka, glucokinase), (2) glucose-6-phosphate dehydrogenase (Glc-6PDH), (3) 6-phosphogluconolactonase, (4) 6-phosphogluconate dehydratase, and (5) 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase.

the intestines of animal hosts where glucuronate derived from glucuronides secreted through the bile duct into the intestines may be an important C-energy source.

Interestingly, the strictly fermentative bacterium *Zymomonas mobilis* is unique in that it uses the ED pathway under anaerobic conditions. In this bacterium, the ED pathway is the only pathway for the catabolism of glucose and other sugars for energy production. *Z. mobilis* utilizes nonoxidative branch of the PPP and an incomplete TCA cycle to synthesize necessary metabolic precursors and intermediates. The pyruvate produced through the ED pathway is ultimately converted to ethanol in *Z. mobilis* (discussed further under ethanol-producing fermentations). The relative inefficiency of the ED pathway in terms of energy production plus the lack of electron transport system enzymes requires that this bacterium be able to rapidly take up and metabolize sugars in its environment in order to achieve and maintain significant growth rates. This is accomplished by maintaining high levels of the ED pathway, and other enzymes needed for C-energy source utilization as well as a high-velocity-facilitated diffusion glucose uptake system. Given this, it is not surprising that *Z. mobilis* lives on plants producing sugar-rich saps. The high rate of ethanol production from sugars also makes *Z. mobilis* a desirable microbe for the commercial production of ethanol.

Pentose phosphoketolase pathway

The pentose phosphoketolase (PPK; aka, heterolactic) pathway is a major route of glucose catabolism in heterolactic or heterofermentative lactic acid-producing bacteria including: species of *Leuconostoc* (e.g., *L. mesenteroides*), *Lactobacillus* (e.g., *L. brevis*, *L. lysopersici*, and *L. pentoaceticus*), *Streptococcus*, *Lactococcus*, *Pediococcus*, *Microbacterium*, *Bacillus*, *Acetobacter* (*A. aceti*), and the mold *Rhizopus*. The heterofermentative lactic acid-producing bacteria typically produce equimolar amounts of CO_2 plus lactate and ethanol or acetate from the catabolism of glucose. Originally the production of these compounds from glucose catabolism was thought to be a result of alternative metabolic routes for pyruvate formed in the EMP pathway. Carbon isotope studies to examine the distribution of the carbon atoms of glucose in the ultimate products of its catabolism indicated that these microbes possessed an alternative pathway of glucose degradation.

The PPK pathway also represents an offshoot of the oxidative branch of the PPP (**Figure 5**). The first five steps in this pathway are shared with the PPP and involve the conversion glucose to xylulose-5-phosphate. Xylulose-5P is then cleaved to form the C_3 compound GAP and two-carbon (C_2) compound acetyl-phosphate by the enzyme pentose (or xylulose-5-phosphate) phosphoketolase. GAP is further catabolized to pyruvate through the triose

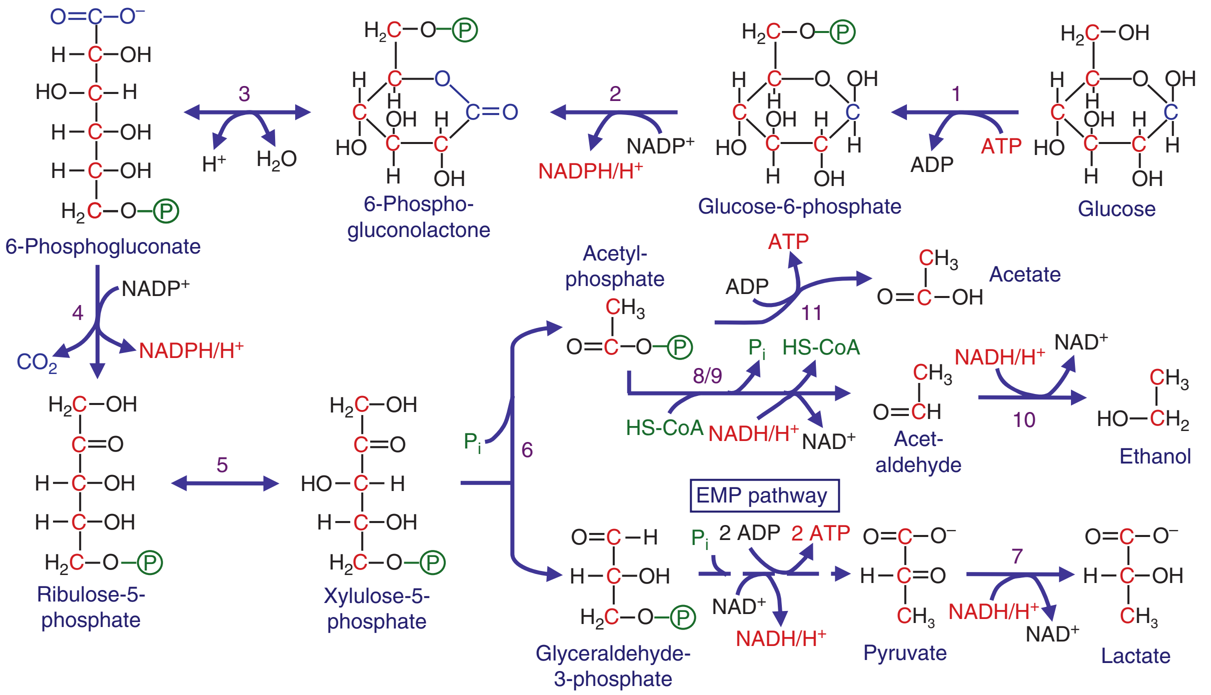

Figure 5 Pentose phosphoketolase (PPK) pathway. Enzymes catalyzing each step are as follows: (2) Glucose-6-phosphate dehydrogenase (Glc-6PDH), (3) 6-phosphoglucono-lactonase, (4) 6-phosphogluconate dehydrogenase, (5) ribulose-5-phosphate epimerase, (6) pentose phosphoketolase (PPK), (7) lactate dehydrogenase (LDH), (8) phosphotransacetylase, (9) (CoA-dependent) aldehyde dehydrogenase, (10) alcohol dehydrogenase, and (11) acetyl phosphate kinase.

phosphate portion of the EMP pathway generating two ATP and one NADH/H^+. The pyruvate is then reduced to lactate by the enzyme lactate dehydrogenase (LDH) utilizing one NADH/H^+. The acetyl-phosphate that is formed can be reductively dephosphorylated to acetaldehyde in a CoA-dependent manner by phosphotransacetylase and aldehyde dehydrogenase. Acetaldehyde is then reduced to ethanol by alcohol dehydrogenase using NADH/H^+ and regenerating NAD^+. Therefore, the conversion of acetyl-phosphate to ethanol generates two NAD^+ molecules from two NADH/H^+ molecules, contributing to the maintenance of O–R cofactor balance. Alternatively, under conditions where NAD^+ can be regenerated without ethanol formation, acetyl-phosphate plus ADP can be converted to acetate plus ATP in a reaction catalyzed by acetyl-phosphate kinase; this allows for the formation of an additional ATP through the PPK pathway. Thus, a single glucose molecule catabolized through the PPK pathway to CO_2, lactate, and acetate can generate a net of two NAD(P)H/H^+ molecules and two ATP.

Unlike for heterofermentative lactic acid bacteria, the PPK pathway seems to be more important for the growth of many types of yeast on xylose (possibly other pentoses) rather than glucose. In these yeasts, little to no pentose phosphoketolase activity was detected when they were grown on glucose. In contrast, pentose phosphoketolase activity increased greatly (~70-fold) when they were grown on xylose as the sole carbon-energy source. This suggested that the PPK pathway is a major means of pentose catabolism in these microbes.

Deviations in the Pathways of Glycolysis

Glycolytic pathways in the Archaea

The Archaea are a relatively newly discovered diverse domain of prokaryotic microbes that can be found in virtually every environment on earth from hot/thermal springs to salt lakes to the open ocean to soils. One group of these bacteria, the saccharolytic archaea, is heterotrophic, living on a variety of saccharides and sugars. Although the central intermediary metabolic pathways of bacteria and eukaryotes are by and large conserved, the saccharolytic archaea have developed sugar-degrading pathways, that is, the Embden–Meyerhof–Parnas and the ED pathways, possessing a varying combination of classical bacterial/eukaryotic glycolytic enzymes and enzyme activities unique to the archaeons. Some utilize only a modified EMP pathway or ED pathway for the catabolism of sugars, while others use a modified EMP pathway for the catabolism of some sugars and a modified ED pathway for the catabolism of other sugars.

The anaerobic thermophilic *Pyrococcus furiosus*, one of the best-studied archaea in terms of its EMP pathway, and many other archaeons possess only four of the ten glycolytic enzymes found in bacteria and eukaryotes, for example, triose phosphate isomerase, phosphoglycerate mutase, enolase, and pyruvate kinase. The other steps of this pathway are catalyzed by enzymes novel to this and other archaeons (**Figure 6**(**a**)). In addition, several of the archaeal glycolytic enzymes show diversity among the archaeons themselves. The enzyme responsible for the phosphorylation of glucose in *P. furiosus* is an ADP-dependent hexokinase (ADP-HK) that is unrelated to the ATP-dependent hexokinase of bacteria and eukaryotes. However, like the bacterial/eukaryote HK, the archaeal ADP-HK exhibits broad substrate (hexose) specificity. An ADP-HK is also present in other *Pyrococcus* species as well as *Thermococcus* species and *Archaeoglobus fulgidus* strain 7324. In comparison, ATP-HK homologues are found in other archaea including *Desulfurococcus amylolyticus*, *Pyrobaculum aerophilum*, *Thermoproteus tenax*, *Thermoplasma volcanium*, *Thermoplasma acidophilum*, and *Aeropyrum pernix*. The phosphoglucose isomerases (PGI) of some archaeons appear to be different, as well, based on genetic analysis. The deduced PGI enzymes of *Pyrococcus*, *Thermococcus*, and *Methanosarcina* species belong to the cupin superfamily of proteins in contrast to the classical PGIs of bacteria and eukaryotes. The archaeal phosphofructokinases (PFK) are found as three types. The first type is found in the hyperthermophiles *P. aerophilum* and *A. pernix*, and appears to be a homologue of the classical ATP-dependent PFK (ATP-PFK) of bacteria and eukaryotes. The PFK of *A. pernix*, the first ATP-PFK characterized from a hyperthermophile, is not allosterically regulated in contrast to most bacterial/eukaryotic PFKs. The second type of PFK enzymes is ADP-dependent (ADP-PFK), and is found in *P. furiosus*, the related *Thermococcus illogic*, the nonsaccharolytic *Methanocaldococcus jannaschii*, the sulfate-reducing *A. fulgidus* strain 7324 and in both thermophilic and mesophilic glycogen-degrading methanogenic archaeons. Sequence analyses of several ADP-PFKs suggest that they are paralogous to the ADP-HKs described above. Furthermore, both the ADP-PFK and ADP-HK enzymes look to be unrelated to the corresponding ATP-PFK/HK enzymes of bacteria and eukaryotes. The third type of PFK enzymes is pyrophosphate (PP_i)-dependent (PP-PFK). This type of PFK enzyme is found in *T. tenax*. PP-PFK enzymes are also found in some bacteria and eukaryotes, and are distantly related to ATP-PFK enzymes. One of the most conspicuous differences observed in Archaea compared to bacteria and eukaryotes is in the conversion of GAP to 3-phosphoglycerate. In bacteria, eukaryotes, and some archaea, this requires two different enzymes (GAPDH and PGK) and generates a NADH/H^+ and one ATP. In *P. furiosus*, a distinctive enzyme catalyzes the single-step (inorganic) phosphate-independent conversion of GAP into 3-phosphoglycerate. This enzyme is the tungsten-containing glyceraldehyde-3-phosphate ferridoxin oxidoreductase (GAPOR). No NADH/H^+ or ATP is generated in this process. In comparison, *T. tenax*

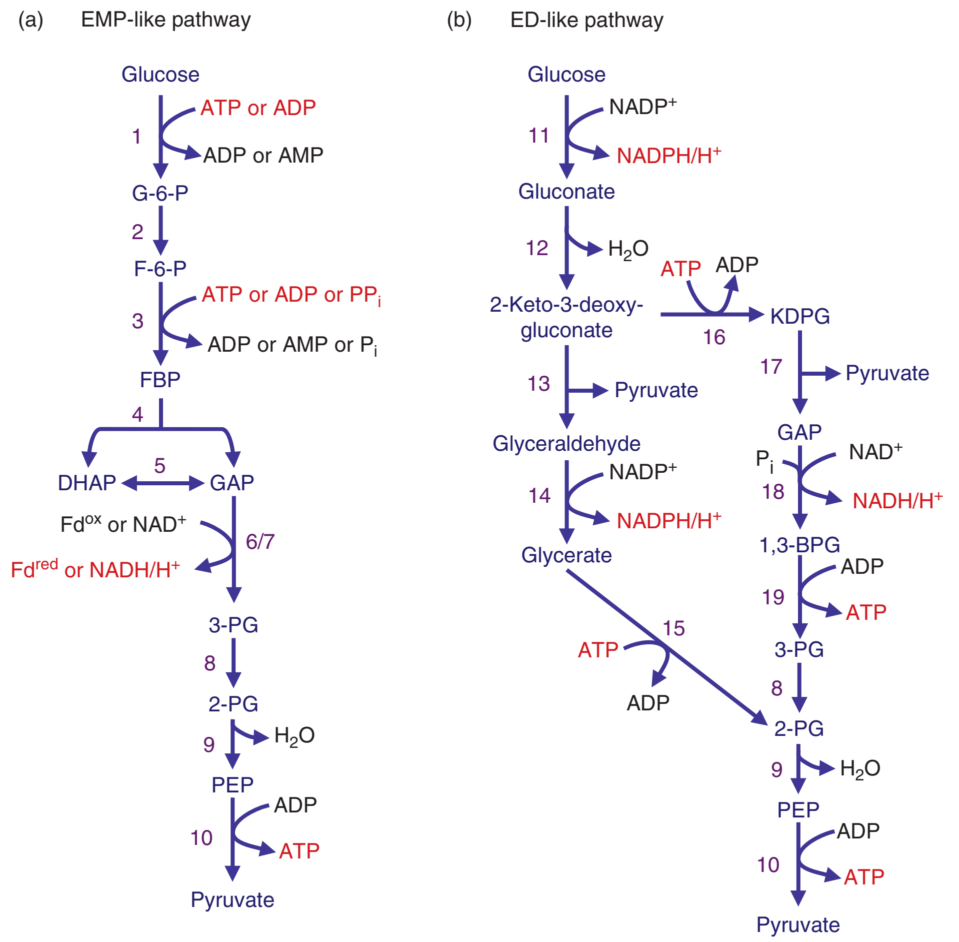
(a) EMP-like pathway
Glucose
ATP or ADP
1
ADP or AMP
G-6-P
2
F-6-P
ATP or ADP or PP_i
3
ADP or AMP or P_i
FBP
4
5
DHAP
GAP
Fd^{ox} or NAD^+
6/7
Fd^{red} or $NADH/H^+$
3-PG
8
2-PG
9
H_2O
PEP
ADP
10
ATP
Pyruvate
(b) ED-like pathway
Glucose
$NADP^+$
11
$NADPH/H^+$
Gluconate
12
H_2O
2-Keto-3-deoxy-gluconate
ATP
ADP
16
KDPG
13
Pyruvate
17
Pyruvate
Glyceraldehyde
GAP
P_i
NAD^+
18
$NADH/H^+$
$NADP^+$
14
$NADPH/H^+$
Glycerate
1,3-BPG
ADP
19
ATP
15
ATP
ADP
3-PG
8
2-PG
9
H_2O
PEP
ADP
10
ATP
Pyruvate

Figure 6 Glycolytic pathways in the Archaea. (a) Embden–Meyerhof–Parnas (EMP)-like pathways present in some Archaea. (b) Entner–Doudoroff (ED)-like pathways present in some Archaea. See text for further explanations and abbreviations of intermediates/molecules in pathways. Fd^{ox}, oxidized ferredoxin; Fd^{red}, reduced ferredoxin. Enzymes catalyzing each step are as follows: (a) (1) ATP-dependent or ADP-dependent hexokinase (ATP-HK or ADP-HK), (2) phosphoglucoisomerase (PGI), (3) ATP-dependent or ADP-dependent or PP_i-dependent phosphofructokinase (ATP-PFK or ADP-PFK or PP_i-PFK), (4) fructobisphosphate aldolase (FBA), (5) triose-phosphate isomerase, (6) glyceraldehyde-3-phosphate ferridoxin oxidoreductase (GAPOR) (7) phosphate-independent NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN), (8) phosphoglycerate mutase (PGM), (9) (phosphoglycerate) enolase, (10) pyruvate kinase (PYK). (b) (11) glucose dehydrogenase/gluconolactonase, (12) gluconate dehydratase, (13) 2-keto-3-deoxy-gluconate (KDG) aldolase; (14) glyceraldehyde dehydrogenase, (15) glycerate kinase, (16) KDG kinase, (17) KDPG aldolase, (18) (phosphate-dependent NAD^+-dependent) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (19) phosphoglycerate kinase.

possesses an allosterically controlled phosphate-independent (or nonphosphorylating) NAD^+-dependent GAP dehydrogenase activity (GAPN), which catalyzes the single-step oxidation of GAP to 3-phosphoglycerate. Here one $NADH/H^+$ is produced but no ATP is generated.

The ED pathways of Archaea are found to vary in two important ways (**Figure 6**(**b**)). The first variant is exemplified by the 'partially phosphorylated' ED-like pathway found in members of the halophilic archaea *Halobacterium*, *Haloferax*, and *Halococcus*. In these archaea, glucose is oxidized to gluconate by glucose dehydrogenase (producing $NADPH/H^+$) and gluconolactonase. Gluconate is converted to 2-keto-3-deoxygluconate (KDG) by gluconate dehydratase. KDG is then phosphorylated by the enzyme KDG kinase, using ATP, to form KDPG. The KDPG is then split by KDPG aldolase to pyruvate and GAP. GAP can then be further metabolized to pyruvate using the enzymes of the triose phosphate portion of the EMP pathway. The phosphorylation of the six-carbon KDG allows for the net production of one ATP via substrate-level phosphorylation during the complete conversion of one glucose to two pyruvates. The second variant of the bacterial ED pathway is present in species of the thermophiles *Thermoproteus*, *Thermoplasma*, and *Sulfolobus*. These archaeons use a 'nonphosphorylated' ED-like pathway in which neither glucose nor the other C_6 intermediates (i.e., gluconate or KDG) are phosphorylated. In this variant, glucose is converted to KDG as described above;

however, KDG (rather than KDPG) is split by KDG aldolase to yield pyruvate and glyceraldehyde. Glyceraldehyde is then oxidized to glycerate by the enzyme glyceraldehyde dehydrogenase generating one NADPH/H^+ from $NADP^+$. Glycerate kinase then phosphorylates glycerate using ATP to form 2-phosphoglycerate (2-PG). 2-PG is then converted to pyruvate by the EMP pathway enzymes enolase and pyruvate kinase, the latter reaction generating one ATP. Since one ATP is used in the phosphorylation of the three-carbon glycerate, there is no net production of ATP through this variant of the ED pathway. However, both of these ED-like pathways potentially yield two NAD(P)H/H^+.

Evidence to date suggests that Archaea do not catabolize glucose via the PPP. However, like bacteria and eukaryotes, they do require the ability to generate pentose intermediates for nucleotide and amino acid biosynthesis. Some archaea such as *Sulfolobus* are capable of growth on pentoses. Together, these suggest that at least some of PPP enzymes may be present in the archaea. Based on genomic analysis, transketolase, ribulose-5-phosphate epimerase, and ribose-5-phosphate isomerase are present in many archaea.

A novel glycolytic pathway has been proposed in *Thermococcus zilligii* based on radio-labeled glucose experiments. In this pathway, a C_6 intermediate (possibly gluconate 6-phosphate) is converted to a five-carbon (C_5) pentose phosphate intermediate (e.g., xylulose 5-phosphate) and formate, by a novel type of lyase. The pentose phosphate is presumably further catabolized via a pentose phosphoketolase pathway similar to that of lactic acid-producing bacteria. This pathway appears to be more important when *T. zilligii* grows on amino acids (e.g., tryptone) since the enzymes involved are repressed by the presence of glucose in the medium.

Methylglyoxal bypass of glycolytic pathways

The methylglyoxal (MG) bypass pathway provides an alternative means for the catabolism of GAP to pyruvate in many bacteria, including *E. coli*, other enterobacteria, and *Pseudomonas* spp., along with yeast (e.g., *S. cerevisiae*) and halophilic Archaea. In this pathway, GAP is converted to pyruvate through the intermediates DHAP, MG (a highly toxic 2-oxo-aldehyde), and lactate, with no generation of ATP. This series of reactions bypasses the energy-yielding steps of the triose phosphate portion of the EMP pathway, in particular the phosphorylating step mediated by GAPDH (**Figure 2**). A proposed physiologic role for this bypass is to allow for the conversion of GAP to pyruvate under phosphate-limiting conditions when the activity of GAPDH would be diminished by the scarcity of one of its substrates, inorganic phosphate. Although no ATP is generated during the MG bypass, the subsequent catabolism of pyruvate may lead to ATP generation.

The MG bypass pathway of the yeast *S. cerevisiae* is presented in **Figure 7**. In the first step, DHAP formed from the splitting of fructose-1,6-bisphophosphate or isomerization of GAP is converted to MG by MG synthase. MG can be converted to lactate through either (1) lactaldehyde by the actions of MG reductase and lactaldehyde dehydrogenase (LALDH) oxidizing one NADPH/H^+ and reducing one NAD^+ in the process or (2) *S*-lactoyl-glutathione via the actions of glyoxalase I and II using reduced glutathione. Lactate is then converted to pyruvate through the reverse reaction of LDH forming one NADH/H^+.

Fates of Pyruvate and Reduced NAD/NADP Formed in Glycolytic Pathways

Pyruvate formed from the various glycolytic pathways described above represents the key intermediate from

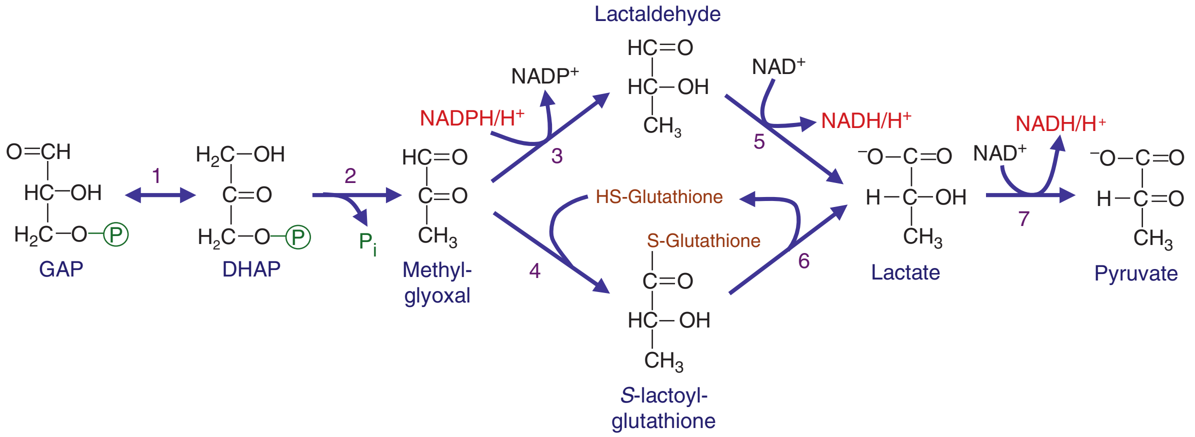

Figure 7 Methylglyoxal (MG) bypass pathway of *Saccharomyces cervisiae*. See text for further explanations and abbreviations of intermediates/molecules in pathways. Enzymes catalyzing each step are as follows: (1) triose phosphate isomerase, (2) MG synthase, (3) MG reductase, (4) glyoxalase I, (5) lactaldehyde dehydrogenase (LALDH), (6) glyoxalase II, and (7) lactate dehydrogenase (LDH).

which a plethora of metabolic by-products (e.g., alcohols, aldehydes, gases) and precursors (e.g., amino acids) are formed. The fate of pyruvate is determined by the environmental conditions in which the microbe finds itself (e.g., aerobic vs. anaerobic environs) as well as the metabolic capabilities of the microbe (e.g., strictly fermentative vs. strictly oxidative metabolisms). Another consideration is how the reduced NAD or NADP formed in several steps of various glycolytic pathways is oxidized so as to restore and maintain the O–R balance to cellular metabolism. This, like the fate of pyruvate, is dependent upon the environmental growth conditions and the basic metabolic capabilities of the microbe.

Aerobiosis – strict aerobes and facultative anaerobes

Growth in the presence of oxygen provides a more effective way of energy/ATP production from glucose; however, it also increases the generation of toxic metabolites such as reactive oxygen species (e.g., superoxide anion, hydrogen peroxide) that must be detoxified. Under aerobic growth conditions, microbes capable of aerobic or oxidative metabolism convert the pyruvate formed from glycolytic pathways into acetyl-CoA and CO_2, reducing NAD^+ to $NADH/H^+$ in the process. This conversion is mediated by pyruvate dehydrogenase (PDH) complex using thiamine pyrophosphate (TPP), lipoate, and coenzyme A (CoA-SH)) as cofactors. This enzyme complex is present in (strict or facultative) aerobic microbes but not in strict anaerobes. In *E. coli*, *Salmonella*, and other bacteria, this complex is encoded by *aceE* (PDH), *aceF* (dihydrolipoamide acetyltransferase), and *lpd* (lipoamide dehydrogenase). The acetyl-CoA formed is further oxidized to two CO_2 through the tricarboxylic acid (TCA; aka, citric acid or Kreb's) cycle, generating one ATP, three additional $NADH/H^+$, and one $FADH_2$ (**Figure 8**).

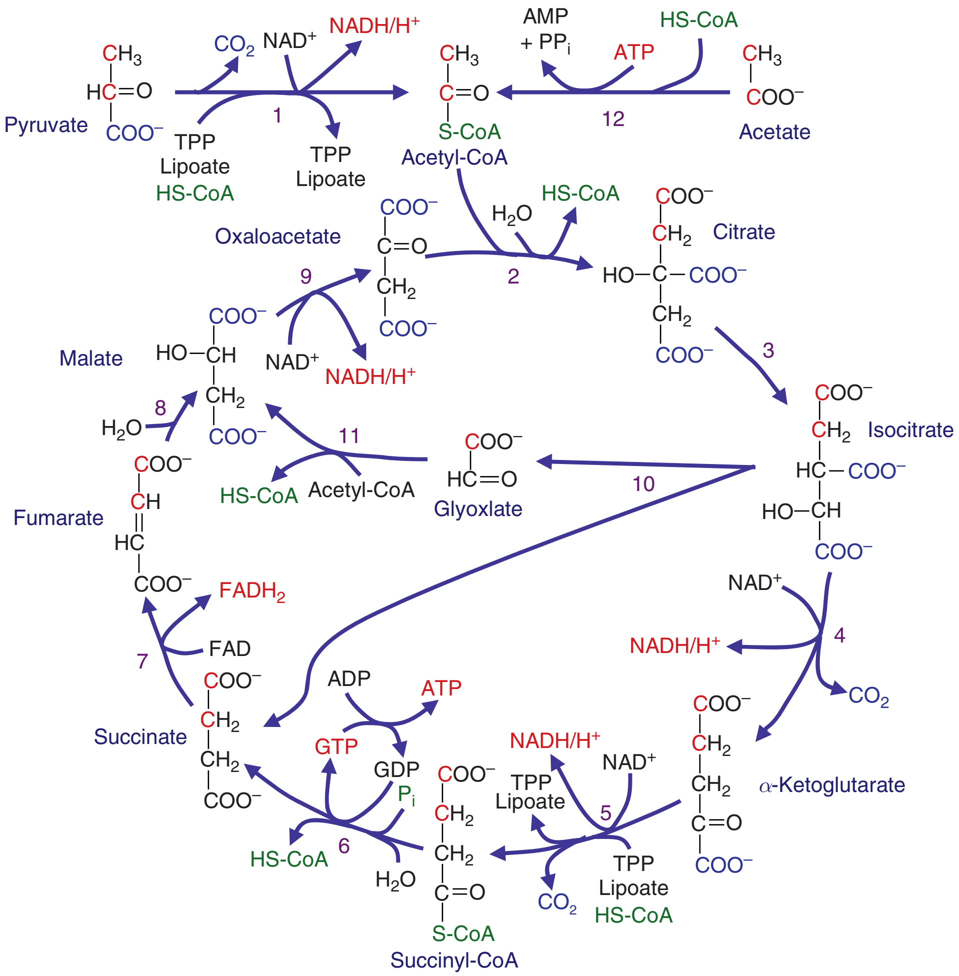
Pyruvate
CH3
HC=O
COO−
CO2
NAD+
NADH/H+
1
TPP
Lipoate
HS-CoA
TPP
Lipoate
CH3
C=O
S-CoA
Acetyl-CoA
AMP
+ PPi
ATP
HS-CoA
12
CH3
COO−
Acetate
Oxaloacetate
COO−
C=O
CH2
COO−
H2O
HS-CoA
2
Citrate
COO−
CH2
HO−C−COO−
CH2
COO−
9
Malate
COO−
HO−CH
CH2
COO−
NAD+
NADH/H+
3
Isocitrate
COO−
CH2
HC−COO−
HO−CH
COO−
8
H2O
11
COO−
HC=O
Glyoxlate
10
HS-CoA
Acetyl-CoA
Fumarate
COO−
CH
HC
COO−
FADH2
FAD
7
NAD+
4
NADH/H+
CO2
Succinate
COO−
CH2
CH2
COO−
ADP
ATP
GTP
GDP
Pi
6
HS-CoA
H2O
NADH/H+
NAD+
TPP
Lipoate
5
α-Ketoglutarate
COO−
CH2
CH2
C=O
COO−
Succinyl-CoA
COO−
CH2
CH2
C=O
S-CoA
CO2
TPP
Lipoate
HS-CoA

Figure 8 Tricarboxylic acid (TCA) and glyoxylate (shunt) cycles. Enzymes catalyzing each step are as follows: (1) pyruvate dehydrogenase complex, (2) Citrate synthase, (3) aconitase, (4) isocitrate dehydrogenase (ICD), (5) α-ketoglutarate dehydrogenase (α-KDH) complex, (6) succinyl-CoA synthetase, (7) succinate dehydrogenase (SDH), (8) fumarase, (9) malate dehydrogenase, (10) isocitrate lyase (ICL), (11) malate synthase and (12) acetyl-CoA synthetase.

Bacillus species have also been shown to produce acetoin, diacetyl, and 2,3-butanediol from pyruvate under aerobic conditions. This process is similar to that described below under mixed acids-producing fermentations (**Figure 9**). However, the purpose of acetoin production in *Bacillus* species under these conditions is quite different. Here it produced as a carbon-energy storage molecule that can be secreted from the cell for future use as cells move into stationary phase due to depletion of more preferred carbon-energy sources. The fact that acetoin is neutral means that its secretion into the growth medium has little effect on extracellular pH.

Tricarboxylic acid cycle

The first reaction in the TCA cycle is the condensation of acetyl-CoA (C_2) and oxaloacetate (OAA; C_4) to form citrate (C_6), which is mediated by the enzyme citrate synthase (encoded by *gltA* in *E. coli* and *Salmonella* or *citZ* in *B. subtilis*).

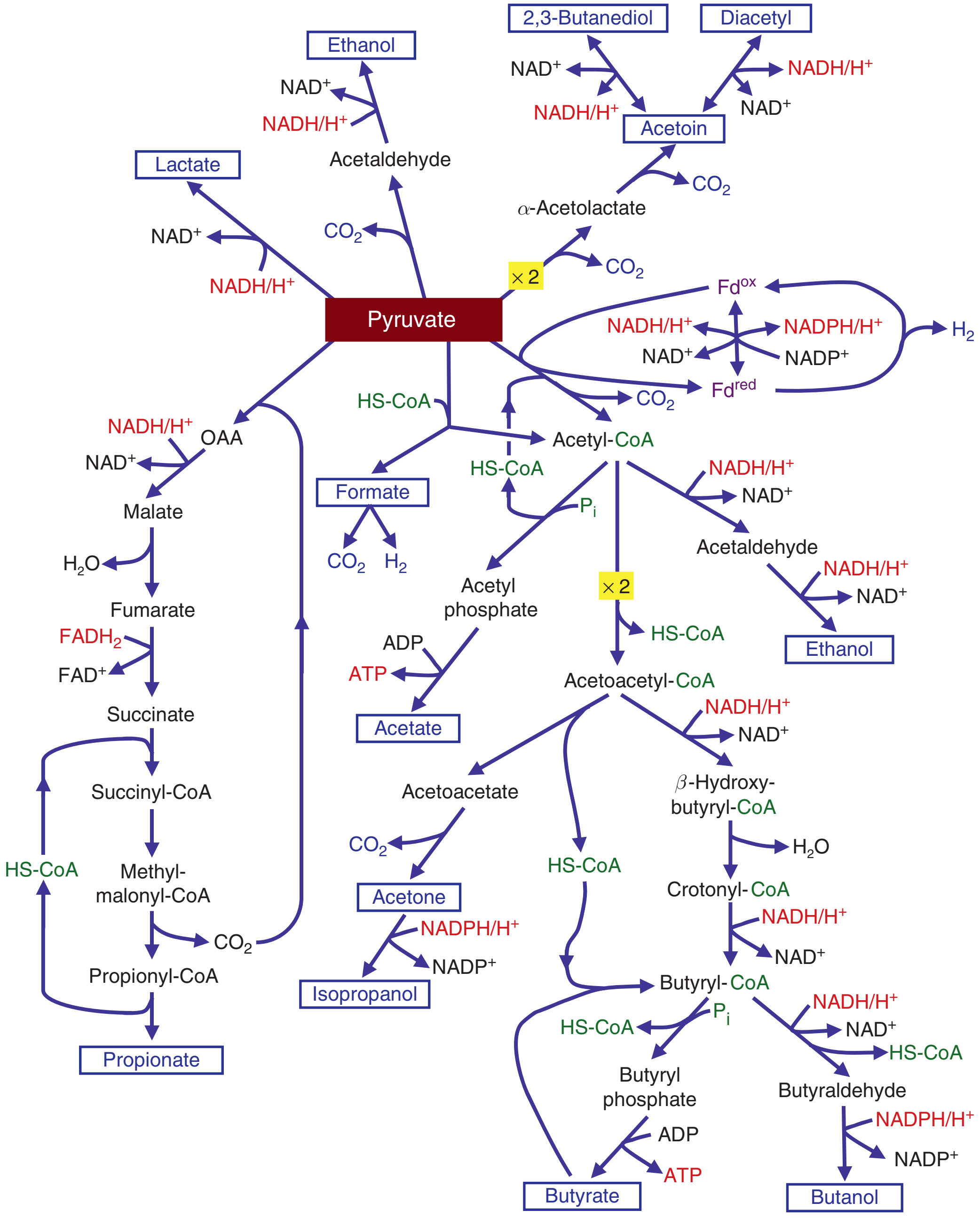
2,3-Butanediol
Diacetyl
Ethanol
NAD+
NADH/H+
NADH/H+
NAD+
NAD+
NADH/H+
Acetoin
Acetaldehyde
Lactate
CO2
α-Acetolactate
NAD+
CO2
×2
CO2
NADH/H+
Pyruvate
Fdox
NADH/H+
NADPH/H+
H2
NAD+
NADP+
HS-CoA
CO2
Fdred
NADH/H+
OAA
NAD+
Acetyl-CoA
HS-CoA
NADH/H+
NAD+
Formate
Malate
Pi
Acetaldehyde
H2O
CO2
H2
Acetyl phosphate
NADH/H+
NAD+
Fumarate
×2
FADH2
ADP
HS-CoA
Ethanol
FAD+
ATP
Acetoacetyl-CoA
Succinate
Acetate
NADH/H+
NAD+
Succinyl-CoA
Acetoacetate
β-Hydroxy-butyryl-CoA
H2O
HS-CoA
Methyl-malonyl-CoA
CO2
HS-CoA
Acetone
Crotonyl-CoA
NADPH/H+
NADH/H+
CO2
NAD+
Propionyl-CoA
NADP+
Isopropanol
Butyryl-CoA
NADH/H+
Pi
HS-CoA
NAD+
HS-CoA
Propionate
Butyryl phosphate
Butyraldehyde
NADPH/H+
ADP
NADP+
ATP
Butyrate
Butanol

Figure 9 Examples of pathways of pyruvate fermentation in microorganisms. The yellow box with ×2 inside indicates that two molecules of the substrate, that is, pyruvate or acetyl-CoA, are combined or condensed in the reaction to yield the indicated product. Fd^{ox}, oxidized ferredoxin; Fd^{red}, reduced ferredoxin. Organic end products of fermentations are boxed and shown in blue while intermediates are shown in black. See the text for enzymes mediating the various reactions presented.

Citrate is then isomerized to isocitrate (C_6) through the unstable intermediate *cis*-aconitate by the enzyme aconitase (encoded by encoded by *citB* in *B. subtilis*). Isocitrate is then oxidized and decarboxylated to α-ketoglutarate (or 2-oxoglutarate; C_5) generating one NADH/H^+ and CO_2. This reaction is mediated by the enzyme isocitrate dehydrogenase (encoded by *icd* in *E. coli* and *Salmonella* or *citC* in *B. subtilis*) through the unstable intermediate oxalosuccinate (C_6). The α-ketoglutarate (α-KG; C_5) produced is then oxidatively decarboxylated to succinyl-CoA by the α-ketoglutarate dehydrogenase (α-KGDH) complex using TPP, lipoate, and CoA as cofactors; in the process one NAD^+ is reduced to one NADH/H^+. This complex is functionally comparable to the PDH complex that catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA. Components of the α-KGDH complex are encoded by the *sucA* (α-KGDH), *sucB* (dihydrolipoamide succinyltransferase), and *lpd* (shared component with PDH complex) in *E. coli* and *Salmonella* or *odhA* (2-oxoglutarate dehydrogenase) in *B. subtilis.*

In the next step, succinyl-CoA synthetase (encoded by *sucD* and *sucC* in *E. coli*, *Salmonella*, and other bacteria) catalyzes the replacement of the CoA in succinyl-CoA with an inorganic phosphate group, forming a transient high-energy phosphate bond that gets transferred to GDP forming GTP; in bacteria, the GTP is used to phosphorylate ADP to yield one ATP. The product of this reaction is succinate (C_4). Succinate is then oxidized to fumarate by the enzyme succinate dehydrogenase (SDH; encoded by *sdhABCD* in *E. coli*, *Salmonella*, and *B. subtilis*). SDH is a flavoprotein and utilizes FAD rather than NAD^+ in its oxidation of succinate to fumarate (C_4); thus, a $FADH_2$ is formed rather than a NADH/H^+. The enzyme fumarase (encoded by *fumC* in *E. coli* and *Salmonella* or *citG* in *B. subtilis*) then adds a water molecule to fumarate producing malate (C_4). In the last step of the cycle, malate is oxidized to OAA by malate dehydrogenase (encoded by *mdh* in *E. coli* and *Salmonella* or *citH* in *B. subtilis*), reducing NAD^+ to NADH/H^+ in the reaction. This regenerates OAA for another round of the cycle.

The NADH/H^+ and $FADH_2$ (generated from the TCA cycle, glycolysis of glucose via the EMP pathway, and conversion of pyruvate to acetyl-CoA) can all be oxidized to NAD^+ and FAD^+, respectively, via the donation of H^+ atoms and transfer of electrons into the electron transport system of the bacteria. This not only helps to maintain oxidation–reduction or O–R balance in the cell but allows for the generation of additional ATP by coupling electron transport, and ultimate reduction of O_2 to H_2O, to ATP production by the F_1F_0-ATPase; a process known as oxidative phosphorylation. This is accomplished by pumping H^+ atoms out of the cytoplasm at key steps along the electron transfer chain to create a proton motive force (pmf) across the membrane that is then utilized by the F_1F_0-ATPase to generate ATP from ADP + P_i as it moves the H^+ back into the cytoplasm. For every pair of electrons transferred from NADH/H^+ up to 3 ATP molecules can be formed; since $FADH_2$ donates electrons further down the electron transport chain it appears to generate only two ATP. Thus, aerobic or facultatively anaerobic microbes growing aerobically on glucose can potentially generate up to 36 ATP, via both substrate-level and oxidative phosphorylation, for every glucose molecule catabolized through the EMP pathway and TCA cycle. This makes oxidative metabolism, by far, more energy efficient for the cell.

The use of O_2 as the terminal electron acceptor for the electron transport system is referred to as aerobic respiration. However, in the absence of O_2 some microbes are able to use alternative electron acceptors whose ultimate reduction can also be coupled to the production of ATP. This process is referred to as anaerobic respiration. One such alternative electron acceptor commonly used by facultative anaerobes (e.g., *E. coli*, *Salmonella*, and other enterobacteria) as well as strict aerobes (e.g., *Pseudomonas* spp., *Bacillus* spp.) is nitrate. Nitrate reduction to nitrite is primarily carried out by an oligomeric protein complex encoded for by the *narGHIJ* operon in *E. coli*, *Salmonella*, and other bacteria; however, many bacteria possess additional nitrate reductase activities that appear to function under different conditions. The primary NarGHJ nitrate reductase activity is expressed only in the absence of O_2 and in the presence of nitrate.

Glyoxylate shunt or cycle

A variation of the TCA cycle occurs when some bacteria, yeasts, and other microbes grow aerobically on acetate as a sole carbon source. Acetate is excreted when *E. coli* and other microorganisms grow rapidly on glucose and can therefore be used as a secondary carbon-energy (C) source when glucose (or other more rapidly utilized C-sources) is exhausted. Acetate can be 'activated' by the addition of CoA forming acetyl-CoA, using the energy released from the hydrolysis of ATP to AMP plus pyrophosphate (PP_i); this reaction is catalyzed by the enzyme acetyl-CoA synthetase (**Figure 8**). The acetyl-CoA then condenses with OAA to form citrate, which is then converted to isocitrate as described above. When preferred C-sources are limited or absent under aerobic conditions, two enzyme activities are expressed: isocitrate lyase (ICL) and malate synthase. These two enzyme activities compose the glyoxylate shunt or cycle (**Figure 8**). In the first reaction, isocitrate is cleaved to form the C_2 molecule glyoxylate and the C_4 molecule succinate by the enzyme ICL. The glyoxylate formed is then combined with acetyl-CoA to form malate (C_4) by the enzyme malate synthase. An additional malate is formed from the succinate produced in the ICL-mediated step using TCA cycle enzymes (**Figure 8**). The glyoxylate cycle, thus, allows for the net production of two malates, one of which can be utilized to replace the OAA used in the citrate synthase reaction while the other can be used for gluconeogenesis (described later) to allow for the generation of other carbon backbone intermediate/precursors.

The yeast *S. cerevisiae* utilizes propionyl-CoA (a C_3 molecule) through a similar cyclic pathway. In the initial steps, propionyl-CoA and OAA are combined to form 2-methylcitrate. 2-Methylcitrate is converted to 2-methylisocitrate, which is then cleaved by the mitochondrial enzyme 2-methylisociterate lyase (encoded by *ICL2)* producing pyruvate and succinate. This cycle appears to be involved in the catabolism of certain amino acids; for example, propionyl-CoA is formed from the oxidative decarboxylation of the threonine catabolism intermediate, 2-ketoisobutyrate.

Anapleurotic reactions

The TCA cycle is an ancillary metabolic pathway that provides not only reducing equivalents in the form of $NADH/H^+$ and $FADH_2$ but also intermediates/precursors for the biosynthesis of amino acids as well as other essential cell components. Biosynthesis of these building block molecules can drain the TCA cycle of intermediates, reducing the overall activity of the cycle. However, these intermediates can be replenished by various anapleurotic (*Gr.* for 'to fill up') enzyme reactions. Heterotrophic microbes need at least one anapleurotic enzyme to grow aerobically on glucose, other hexoses, or glycolytic intermediates. For example, the production of OAA is required for the continued flow of hexose-derived carbon compounds into the TCA cycle when conditions result in TCA cycle intermediates being rerouted into biosynthetic pathways.

Some anapleurotic enzyme reactions include:

1. PEP carboxykinase:

$$\text{PEP} + CO_2 + H_2O \rightarrow \text{OAA} + P_i$$

2. Pyruvate carboxylase:

$$\text{pyruvate} + CO_2 + \text{ATP} + H_2O \rightarrow \text{OAA} + \text{ADP} + P_i$$

3. Malic enzyme:

$$\text{pyruvate} + CO_2 + \text{NADPH}/H^+ \leftrightarrow \text{L-malate} + \text{NADP}^+$$

It should be noted that the thermodynamics of this reaction favors the reverse reaction, that is, the conversion of malate to pyruvate, limiting the role of malic enzyme in replenishing malate for use in the TCA cycle.

4. Glutamate dehydrogenase:

$$\text{glutamate} + \text{NAD}^+ \rightarrow \alpha\text{-ketoglutarate} + NH_4{}^+ + \text{NADH}/H^+$$

In addition to these reactions, OAA and α-ketoglutarate can also be generated from the deamination of aspartate and glutamate, respectively, by the actions of specific transaminases.

Anaerobiosis – facultative anaerobes and strict anaerobes

The lack of conditions (e.g., presence of O_2) or abilities (e.g., lack of membrane-associated electron transport enzyme systems) that allow for aerobic or anaerobic respiration require facultative and strictly anaerobic bacteria to use pyruvate and/or other organic compounds/intermediates as ultimate electron acceptors producing, to varying degrees, a variety of acids, aldehydes, gases, or alcohols via a process known as fermentation. The function of these fermentative steps is to oxidize NADH or $FADH_2$ to help maintain O–R balance for cellular metabolism and in some cases generate additional energy in the form of membrane pmf or ATP. Many of the products of these fermentation pathways (e.g., ethanol, butanol, acetone, butyric acid, acetic acid, propionic acid, or lactic acid) are of significant economic importance in both the commercial chemical and food industries. Based on the major end product(s) formed in the fermentation reactions, fermentations can be grouped into several types: (1) lactic acid-producing fermentations, (2) butyric acid/butanol–acetone-producing fermentations, (3) propionic acid-producing fermentations, (4) mixed acids-producing fermentations, and (5) ethanol-producing fermentations.

Lactic acid-producing fermentations

As discussed above, homofermentative and heterofermentative lactic acid bacteria, that is, species of *Lactococcus*, *Lactobacillus*, and *Streptococcus* as well as others, primarily produce lactate (homofermentative) or produce lactate along with other products (heterofermentative) from the fermentation of pyruvate. These bacteria convert pyruvate produced from glycolytic pathways to lactate using the enzyme LDH. In the process, pyruvate reduction to lactate is coupled to $NADH/H^+$ oxidation to NAD^+ (**Figure 9**). This reforms the NAD^+ that is needed for the oxidation steps of glycolysis and helps maintain O–R balance in the cell.

Many lactic acid bacteria in the genus *Lactococcus* (*L. cremoris*, *L. lactis*, and *L. diacetylactis*), as well as species of the genera *Leuconostoc* and *Lactobacillus*, also form acetoin, diacetyl, and 2,3-butanediol. The formation of these end products can occur through the condensation of two pyruvates and decarboxylation to form α-acetolactate followed by another decarboxylation step to form acetoin. Acetoin can then form either 2,3-butanediol or diacetyl (discussed further under mixed acids fermentations). These end products can also be formed from acetate through acetyl-CoA-C_2 condensations depending on components in the growth medium. The metabolic properties of lactic acid bacteria play key roles in the commercial production of butter and many fermented dairy products, for example, buttermilk, sour cream, cottage cheese, yogurt, cheddar cheese, and are thus of enormous commercial importance to the dairy-food industry.

Butyric acid/butanol–acetone-producing fermentations

Members of *Clostridium*, *Fusobacterium*, *Eubacterium*, and *Butyrivibrio* as well as several poorly characterized microbes found in a variety of natural environs, for example, marshes, forests, and anaerobic sewage digestion systems, produce butyrate (a.k.a., butanoate) as a major product of pyruvate fermentation. In addition, many produce, to varying degrees, butanol, acetone, isopropanol, acetate, 2,3-butanediol, ethanol, CO_2, and H_2 as end products of fermentation (**Figure 9**).

Clostridium acetobutylicum is a good example of a microbe that produces these products from pyruvate. During its growth on glucose or other sugars, the pyruvate formed through the EMP pathway is converted to acetate and butyrate in what is referred to as the acidogenic phase of growth (**Figure 9**). In the first steps of this conversion, pyruvate is oxidatively decarboxylated forming acetyl-CoA and reduced ferredoxin (Fd^{red}), as opposed to $NADH/H^+$ as is the case of the PDH complex-mediated reaction. In this case, the formation of acetyl-CoA from pyruvate is mediated by the iron–sulfur cluster containing pyruvate–ferredoxin oxidoreductase complex. The reduction of oxidized ferredoxin (Fd^{ox}) to Fd^{red} in this reaction is linked to the oxidation of Fd^{red} by hydrogenase forming H_2 in the process. The Fd^{red} can also be oxidized by a NADPH-ferredoxin oxidoreductase, forming Fd^{ox} and $NADPH/H^+$ under these conditions. The acetyl-CoA has two fates at this point. In one pathway, acetyl-CoA is converted to acetyl phosphate by the addition of inorganic phosphate and concomitant release of CoA in a reaction mediated by phosphotransacetylase. Acetyl phosphate plus ADP is then converted to acetate and ATP, by the enzyme acetate kinase. The production of acetate, CO_2, H_2, and ATP from pyruvate plus inorganic phosphate is referred to as the phosphoroclastic reaction. The phosphoroclastic reaction is prevalent among anaerobic bacteria and provides an additional mechanism for the generation of ATP from sugar fermentation in these bacteria. In an alternative pathway, two acetyl-CoA molecules condense to form acetoacetyl-CoA in a reaction mediated by acetyl-CoA–acetyltransferase. The acetoacetyl-CoA can then be reduced by $NADH/H^+$ to β-hydroxybutyryl-CoA via the action of β-hydroxybutyryl-CoA dehydrogenase. β-Hydroxybutyryl-CoA is in turn converted to crotonyl-CoA plus one H_2O by the enzyme crotonase. Crotonyl-CoA is then reduced by $NADH/H^+$ to butyryl-CoA in a reaction mediated by butyryl-CoA dehydrogenase. Butyryl-CoA can then be converted to butyryl phosphate by exchanging an inorganic phosphate for the CoA moiety in a reaction similar to the formation of acetyl phosphate described above. This reaction is mediated by phosphotransbutyrylase. Butyryl phosphate is converted to butyrate with the concomitant phosphorylation of ADP to form ATP. This provides a second mechanism for the formation of ATP in these anaerobes. Metabolizing pyruvate to acetate and butyrate provides these anaerobes the ability to generate twice the ATP per glucose molecule catabolized than lactic acid-producing bacteria (discussed above) or ethanol-producing yeast and bacteria (discussed below).

As the pH of the culture drops (generally to below a pH of 5) due to the production of acetic acid and butyric acid (as discussed above) and the culture enters into a stationary phase, *C. acetobutylicum*, and many other anaerobes, undergo a metabolic shift. They enter what is known as the solventogenic phase where ‘solvents’ such as acetone, butanol, and possibly isopropanol are produced as metabolic end products, as opposed to acidic end products. In this phase, the acetoacetyl-CoA formed from the condensation of two acetyl-CoA molecules serves as the branch point for the production of these products. In one path, the acetoacetyl-CoA is converted to acetoacetate in a reaction that is coupled to the transfer of the CoA moiety to butyrate (or acetate) to form butyryl-CoA (or acetyl-CoA). The enzyme catalyzing this process is acetoacetyl-CoA:acetate/butyrate:CoA transferase. The acetoacetate formed is decarboxylated to produce acetone plus CO_2 by acetoacetate decarboxylase. Some clostridia (e.g., *C. beijerinickii*) can reduce acetone using $NADPH/H^+$ to isopropanol by a reaction mediated by an isopropanol dehydrogenase activity. In a second path, the acetoacetyl-CoA is converted to butyryl-CoA, as described above for butyrate production. Butyryl-CoA may also be produced by the transfer of CoA from acetoacetyl-CoA to butyrate, which is linked to acetoacetate formation (discussed above). In the solventogenic phase, the butyryl-CoA formed is then reduced by $NADH/H^+$ releasing the CoA moiety forming butyraldehyde in a reaction mediated by butyraldehyde dehydrogenase. Butyraldehyde is then reduced by $NADPH/H^+$ to butanol by a butanol dehydrogenase activity. A couple of features of solventogenic phase metabolism are noteworthy. One is that the ATP yield is significantly reduced compared to the acidogenic phase, since ATP would only be generated during substrate-level phosphorylation reactions in the EMP pathway. Secondly, many of the reactions of butanol–acetone fermentation result in the generation of NAD^+ or $NADP^+$, creating the need to regenerate NADH and NADPH, respectively, in order to maintain O–R balance in the cell. This can be accomplished by the $NADH/H^+$-generating steps of the triose phosphate portion of the EMP pathway as well as the oxidation of Fd^{red} that was produced during the conversion of pyruvate to acetyl-CoA. During the solventogenic phase, the oxidation of Fd^{red} is primarily mediated by two enzyme activities, NADH-ferredoxin oxidoreductase and NADPH-ferredoxin oxidoreductase, as opposed to hydrogenase, which mainly functions during the acidogenic phase. These enzyme activities regenerate NADH and NADPH, respectively, along with Fd^{ox} (**Figure 9**).

Propionic acid-producing fermentations

Members of *Propionibacterium*, *Bacteroides*, *Bifidobacterium*, and *Veillonella*, as well as some species of *Clostridium* and *Corynebacterium*, produce propionate, acetate, and CO_2 as the major end products of glucose, lactate, and glycerol fermentation. In these bacteria, pyruvate is carboxylated to form OAA in a reaction mediated by methylmalonyl-CoA–oxaloacetate transcarboxylase using biotin and coenzyme/vitamin B_{12} as cofactors. This reaction also yields propionyl-CoA. In the next series of reactions, OAA is first reduced to malate by $NADH/H^+$ through the action of malate dehydrogenase. A water molecule is then removed from malate to form fumarate via the action of malate dehydratase/fumarase. Fumarate is then reduced to succinate by $FADH_2$ in a reaction catalyzed by fumarate reductase. Succinate is converted to succinyl-CoA, which is then converted to methylmalonyl-CoA by the coenzyme/vitamin B_{12}-containing methylmalonyl-CoA mutase. The enzyme activity catalyzing the succinate to succinyl-CoA conversion is propionyl-CoA:succinate:CoA transferase. In this reaction, the CoA from propionyl-CoA is transferred to succinate, yielding both succinyl-CoA and propionate (**Figure 9**). These bacteria can also decarboxylate pyruvate to form acetate and CO_2, yielding one ATP and one $NADH/H^+$ in the process. Thus, the formation of acetate plus CO_2 from pyruvate allows for the generation of an additional ATP from sugar catabolism while the conversion of pyruvate to propionate allows for the regeneration of NAD^+ helping to maintain O–R balance in the cell.

In addition to forming propionate, acetate, and CO_2 from sugar fermentation, propionic acid bacteria ferment lactate to acetate, CO_2, and propionate. The overall process produces two propionate, one acetate, and one CO_2 from three lactate molecules with the additional yield of one ATP. *Clostridium propionicum*, *Bacteroides ruminicola*, *Peptostreptococcus* species, and others are able to convert lactate to lactyl-CoA and then to acrylyl-CoA by the removal of a water molecule; the latter step is catalyzed by lactyl-CoA dehydratase. Acrylyl-CoA is then reduced by $NADH/H^+$ to propionyl-CoA by the enzyme acrylyl-CoA reductase. Propionyl-CoA is then converted to propionate. The fermentation properties of propionic acid bacteria make them important in the manufacture of Swiss cheese with propionate and acetate contributing to the unusual flavor and CO_2 production causing the wholes characteristic of these cheeses.

Mixed acids-producing fermentations

Although other bacteria can produce mixtures of acidic end products, mixed acids-producing fermentations are typically associated with members of the Enterobacteriaceae family of Gram-negative bacteria (e.g., species of *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, and *Shigella*). These bacteria typically produce a collection of acetic acid, formic acid, lactic acid, and in some cases succinic acid as fermentative end products of glucose catabolism. In most cases, acetic acid and formic acids are the major end products produced. The production of acidic end products from glucose fermentation is the basis for a positive reaction in the methyl red test used in the laboratory identification of the enterobacteria.

During anaerobiosis, enterobacteria take pyruvate, formed primarily from the EMP pathway of glucose dissimilation, and split it to form formate and acetyl-CoA. This reaction is catalyzed by a CoA-dependent pyruvate–formate lyase (PFL; encoded by the *pfl* gene). The acetyl-CoA formed is then converted to acetyl phosphate by the enzyme phosphotransacetylase (encoded by the *pta* gene). As described above for the butyrate-producing bacteria, phosphoacetyltransferase essentially exchanges the CoA moiety for an inorganic phosphate in this reaction. Acetyl phosphate is then converted to acetate with the concomitant formation of ATP from ADP in a reaction mediated by the enzyme acetate kinase (**Figure 9**). This pathway provides an important mechanism for the generation of additional ATP during anaerobiosis, in these bacteria. The formate that is produced can be further broken down to CO_2 and H_2 by the enzyme complex formate-hydrogen lyase (FHL). FHL complex is composed of a formate dehydrogenase activity (FDH-H; encoded by the *fdhF* gene) that generates the CO_2 and a hydrogenase-3 activity that forms the H_2. This reaction only occurs in the absence of an electron acceptor, that is, oxygen or nitrate, and the presence of formate and the cofactor molybdate. The expression *fdhF* also appears to require acidic pH conditions. PFL expression is dependent upon two EMP enzyme activities, namely phosphoglucoisomerase and PFK. Interestingly, the expression of PFL, FDH-H, and hydrogenase-3 all increase under carbon-energy source starvation.

The enterobacteria can also produce lactate and ethanol under anaerobic conditions. As described above for the lactic acid bacteria, lactate is formed from pyruvate through the action of a (NADH-dependent) lactate dehydrogenase (LdhA; encoded by the *ldhA* gene). Ethanol is formed from acetyl-CoA via the action of a CoA-linked acetaldehyde dehydrogenase, which converts acetyl-CoA to acetaldehyde, and a NADH-dependent alcohol dehydrogenase, which reduces acetaldehyde to ethanol using $NADH/H^+$. Both these fermentation pathways play important roles in the maintenance of O–R balance in the anaerobically growing cells.

Some genera of Enterobacteriaceae (e.g., *Enterobacter*, *Klebsiella*, *Serratia*, and *Erwinia*) as well as other bacteria (e.g., *Lactococcus lactis*) also produce 2,3-butanediol and diacetyl (aka, 2,3-butanedione) from oxidation and reduction, respectively, of acetoin. The first step in this pathway is the condensation of two pyruvate molecules, and concomitant decarboxylation, to form α-acetolactate. This reaction is mediated by a α-acetolactate synthase activity (an enzyme activity that is also important in branched-chain amino acid synthesis). α-Acetolactate is then decarboxylated to form acetoin in a reaction catalyzed by α-acetolactate decarboxylase. Acetoin can be either oxidized to diacetyl by an acetoin dehydrogenase activity (aka, diacetyl reductase), using NAD^+ as an electron acceptor, or reduced by $NADH/H^+$ via the action of acetoin reductase (aka, 2,3-butanediol dehydrogenase). Both of these reactions are reversible. In *Enterobacter aerogenes*, the acetoin dehydrogenase/diacetyl reductase activities appear to regulate the balance between diacetyl and 2,3-butanediol production from acetoin. The production of acetoin by *Klebsiella*, *Enterobacter*, and *Serratia* species is the basis for a positive Voges–Proskauer (VP) test in the laboratory identification of these bacteria.

Ethanol-producing fermentations

Ethanol is produced by a variety of microorganisms as a fermentation end product. For some, ethanol is the primary or only end product of pyruvate metabolism while for others ethanol is produced along with multiple end products. The latter has been discussed above under the lactic acid-, butyric acid/acetone–butanol-, and mixed acids-producing fermentations sections. However, for the yeast *S. cerevisiae* and the bacterium *Z. mobilis* the production of ethanol and CO_2 are the end products of pyruvate catabolism. In these organisms, pyruvate (formed through the EMP pathway in *S. cerevisiae* and ED pathway in *Z. mobilis*) is first decarboxylated to acetaldehyde by the enzyme pyruvate decarboxylase (unusual for bacteria). Acetaldehyde is then reduced to ethanol by $NADH/H^+$ via the action of alcohol dehydrogenase. The production of ethanol by these two organisms is of particular interest to (beer brewing) industry as well as the beer loving public.

Archaea

Pyruvate catabolism is of special importance to the saccharolytic archaea because of the low energy yields from the modified EMP and ED pathways present in these microorganisms. As a result, all or most of their energy (i.e., ATP) must come from the metabolism of the pyruvate formed in these pathways.

All heterotrophic saccharolytic archaea oxidatively decarboxylate pyruvate in a CoA-dependent manner to acetyl-CoA plus CO_2 in a reaction catalyzed by pyruvate–ferredoxin oxidoreductase complex, similar to that described above for anaerobic bacteria. However, a functional Bacteria- or Eukarya-type PDH complex has yet to be described in the Archaea. Homologues, however, have been found in the genomes of *T. acidophilum* and those halophilic Archaea analyzed.

In anaerobic archaea (e.g., species of *Desulfurococcus*, *Pyrococcus*, and *Thermococcus*), acetyl-CoA and ADP plus P_i is converted to acetate and ATP by an ADP-forming acetyl-CoA synthetase activity. The enzyme is named for the reverse reaction and is an enzyme activity unique to Archaea. This reaction allows these archaeons to produce two ATP for every glucose ultimately catabolized to two acetate plus two CO_2. In those archaea that reduce O_2, NO_3^-, or sulfur, the acetyl-CoA formed is oxidized via the TCA cycle to two CO_2. In the process, $NADH/H^+$, $FADH_2$, and GTP are formed as in the Bacteria and Eukarya.

Gluconeogenesis

In order for microorganisms to grow on relatively poor carbon sources such as L-malate (C_4) or succinate (C_4), glycerol (C_3), pyruvate (C_3), lactate (C_3), or acetate (C_2), they must be able to synthesize hexoses (e.g., glucose or fructose phosphates) needed for cell-wall components (e.g., peptidoglycan), carbon-energy storage molecules (e.g., glycogen) as well as pentose moieties required for nucleic acid biosynthesis. The synthesis of glucose from pyruvate is accomplished essentially by reversing the EMP pathway in a process known as gluconeogenesis. Although many of the steps of the EMP pathway are reversible, there are three reactions whose free-energy requirement is so high as to essentially prevent the reverse reaction. Therefore, these steps are catalyzed by distinct enzyme activities.

The first step to overcome is the conversion of pyruvate to PEP. *E. coli*, other enterobacteria, and several Archaea studied accomplish this in the direct synthesis of PEP from pyruvate in the following reaction:

$$\text{pyruvate} + \text{ATP} + H_2O \rightarrow \text{PEP} + \text{AMP} + P_i$$

This reaction is catalyzed by the enzyme PEP synthetase. Fungi and some bacteria (e.g., *Pseudomonas*) achieve the conversion of pyruvate to PEP via the following two-step process:

1. pyruvate + CO_2 + ATP → oxaloacetate + ADP + P_i
2. oxaloacetate + GTP → PEP + CO_2 + GDP

The first reaction is catalyzed pyruvate carboxylase (previously discussed above under ‘Anapleurotic reactions’). The second reaction is catalyzed by (Mg^{2+}-dependent) PEP carboxykinase (encoded by *pckA* in *E. coli*, *Salmonella*, and many other bacteria). The latter step represents the first committed step in gluconeogenesis. This enzyme activity is essential for the cell to use carbon(-energy) compounds that feed into the TCA cycle (e.g., citrate, succinate, or malate) or acetate (via the glyoxylate cycle) as a sole carbon(-energy) source.

The second step of the EMP pathway that must be overcome is the conversion of FBP to fructose-6-phosphate. This is performed by the enzyme fructose-1,6-bisphosphatase (encoded by the *fbp* gene in *E. coli*, *Salmonella*, and many other bacteria) by means of the following reaction:

$$\text{FBP} \rightarrow \text{fructose-6-phosphate} + \text{P}_\text{i}$$

In *E. coli*, *fbp* mutants are unable to grow on succinate, L-malate, glycerol, or acetate as the sole carbon-energy sources. However, these mutants can grow on pentoses since they are able to make hexose phosphates through the generation of GAP.

The third step of the EMP pathway that must be bypassed is the dephosphorylation of glucose-6-phosphate to glucose. The enzyme glucose-6-phosphatase mediates the reaction:

$$\text{glucose-6-phosphate} \rightarrow \text{glucose} + \text{P}_\text{i}$$

Although this enzyme is present, it does not appear to be required for growth on C_4, C_3, or C_2 compounds. One notable consequence of converting pyruvate to glucose in gluconeogenesis is the significant energy expenditure required:

$$\begin{aligned}&2\ \text{pyruvate} + 4\text{ATP} + 2\text{GTP} + 2\text{NADH/H}^+ + 4\text{H}_2\text{O}\\&\rightarrow 1\ \text{glucose} + 2\text{NAD}^+ + 4\text{ADP} + 2\text{GDP} + 6\text{P}_\text{i}\end{aligned}$$

Utilization of Polysaccharides, Oligosaccharides, and Monosaccharides

Microorganisms are remarkable in their ability to utilize a wide variety of carbon compounds as carbon-energy sources. Furthermore, in a mixture of carbon-energy sources, they are typically utilized in an order of preference with those capable of supporting the most rapid growth being used first and the utilization of the others being inhibited until the preferred source is depleted. Glucose is the preferred carbon-energy source for most bacteria and fungi that employ the EMP pathway as their major or sole glycolytic pathway, and when present it represses the utilization of other sources in a process known as glucose catabolite repression. For those microorganisms that utilize alternative glycolytic pathways, such as the ED pathway (e.g., species of *Pseudomonas*, *Agrobacterium*, *Azotobacter*, *Caulobacter*, and *Zymomonas*), as a major or sole glucose dissimilation pathway, other organic compounds typically function as preferred carbon-energy sources. A good example of the latter is the pseudomonads, which prefer organic acids and TCA cycle intermediates as carbon-energy sources, and, when present with glucose prevent the catabolism of glucose.

Utilization of Polysaccharides

Polysaccharides are the most abundant form of carbohydrates present in natural environments. These include the polysaccharides composing the cell walls of plants (e.g., cellulose, pectin, hemicellulose, mannans, xylans), carbon-energy source storage polysaccharides (e.g., starches, glycogen), fungal cell walls (e.g., chitin, mannans, glycans), bacterial cell walls (e.g., peptidoglycan), and exoskeletons of arthropods (e.g., chitin derivatives). The size of these polysaccharides makes these molecules insoluble and virtually unavailable for utilization in these forms. Microbes must therefore secrete extracellular enzymes capable of degrading these polysaccharides to smaller soluble oligo- or monosaccharides that can be transported into cells for utilization as carbon-energy sources.

Plant-derived polysaccharides (e.g., pectin and cellulose) are some of the most abundant and important in the world. Pectin is a highly methylated polymer of D-galacturonic acid connected through β-1,4 linkages while cellulose is a β-1,4-linked glucose polymer; both are major components of plant cell walls. The ability to degrade pectin and cellulose (via the production of pectinases and cellulases) is a virulence factor for many bacterial and fungal plant pathogens, for example, *Erwinia chrysanthemi*, *E. carotovora*, *Cladosporium cucumerinum*, and *Moloninia fructigena*. In addition, degradation of these plant polysaccharides has a significant economic impact since plant-derived feeds are major sources of nutrients for domestic animals (e.g., cattle). The plant pathogens *E. chrysanthemi* and *E. carotovora* degrade pectin by a group of sequentially acting enzymes that first remove methoxyl groups (pectin methylesterase) along the polymer to yield polygalacturonate. The polygalacturonate is then digested by pectate lyases or exopolygalacturonase to produce digalacturonate. Residues are then ultimately catabolized to pyruvate and GAP via the ED pathway within the cell. Bacteria (e.g., *Butyrivibrio fibrisolvens* and *Lachnospira multiparus*) that inhabit the rumen of herbivores are essential to allowing pectin and cellulose from plant-derived feed to be used as nutrients for the animal. Cellulose-degrading microbes (e.g., the fungi *Trichoderma* and *Phanaerochete* and the bacteria *Cellulomonas*, *Microbispora*, *Thermomonaspora*, *Ruminococcus flavofaciens*, *Fibrobacter succinogenes*, *Clostridium cellulovorans*, and *C. cellulolyticum*) produce a set of enzymes, which are typically secreted extracellularly or form a cell surface-associated multiprotein complex (cellulosome), that first degrade the glucose polymer to shorter oligosaccharides (i.e., endo-β-1,4-glucanase). These oligosaccharides are then degraded to the disaccharide cellibiose by exo-β-1,4-glucanase. Cellibiose can be transported into the cell where it is phosphorylated then cleaved to glucose and glucose-6-phosphate by β-glucosidase.

Starch is a very common carbon-energy storage molecule in plants that is typically a mixture of amylose (long unbranched chains of α-1,4-linked glucose) and amylopectin (highly branched α-1,4-linked glucose chains with α-1,6 linkages at branch sites). Glycogen is the primary carbon-energy storage molecule in animals that, like amylopectin, is a highly branched α-1,4-linked glucose polymer with frequent α-1,6-branches. Amylose, amylopectin, and glycogen can all be degraded by α-amylases and β-amylases to glucose, maltose, and a highly branched core dextrin that can be degraded by an α-1,6-glucosidase (debranching enzyme). All three enzymes are produced by a variety of bacteria and fungi. Species of *Aspergillus* (a mold) produce glucoamylases that digest starch to glucose. *Bacillus* species (e.g., *B. polymyxa*, *B. megaterium*, *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*) produce α-amylases and/or β-amylases. The enzymes of *B. amyloliquefaciens*, in particular, produce maltotriose, maltohexaose, and maltoheptaose, which are then transported into the cell and degraded further to glucose. *Pseudomonas stutzeri* and *E. aerogenes* both secrete exoamylases that generate maltotetraose and maltohexaose, respectively. Some species of *Bacillus* (e.g., *B. stearothermophilus*) and *Klebsiella* (e.g., *K. oxytoca* and *K. pneumoniae*) secrete starch-degrading enzymes, cyclodextrin glucosyltransferase, that form cyclodextrins, which are cyclic oligosaccharides composed of six to twelve glucose molecules linked via α-1,4-glycosidic bonds. Cyclodextrins are resistant to digestion by the typical starch-degrading enzymes produced by other microbes; however, the bacteria that generate them can transport the cyclodextrins into the cell using specific uptake systems and degrade them to individual glucose molecules.

Utilization of Oligosaccharides and Monosaccharides

Monosaccharides and relatively short oligosaccharides when present in the growth environment may be taken up into cells through either energy-requiring active transport or group translocation involving phosphorylation, for most microbes. Some microorganisms that grow in high sugar concentration environs, that is, *S. cerevisiae* and *Z. mobilis*, may also use facilitated diffusion to transport sugars into the cell. Monosaccharides entering the cell or released from the degradation of oligosaccharides in the cytoplasm must be phosphorylated to allow for further catabolism and to prevent them from moving out of the cell. The utilization of a particular sugar depends upon whether the microbe possesses the necessary transport system and degradative enzymes.

Disaccharide/oligosaccharide utilizations

E. coli and many other lactose-fermenting microbes possess the *lac* operon that encodes a lactose permease and β-galactosidase, which cleaves the disaccharide lactose to D-galactose and D-glucose. D-Galactose can then be catabolized through either the tagatose pathway or the Leloir pathway. In the tagatose pathway, D-galactose is phosphorylated using ATP to galactose-6-phosphate via the action of HK. Galactose-6-phosphate isomerase converts galactose-6-phosphate to tagatose-6-phosphate, which is phosphorylated using ATP to form tagatose-1,6-phosphate by the enzyme tagatose-1,6-phosphate kinase. Tagatose-1,6-bisphosphate aldolase then splits tagatose-1,6-bisphosphate to yield DHAP and GAP, which can then enter into the EMP pathway. In the Leloir pathway, D-galactose is phosphorylated using ATP to galactose-1-phosphate by galactokinase. Galactose-1-phosphate is then converted to glucose-1-phosphate by phosphogalactose uridyltransferase. Glucose-1-phosphate is then converted to glucose-6-phosphate by phosphoglucomutase. Glucose-6-phosphate can then enter into the EMP pathway for further catabolism. In *L. casei* and some other microbes, lactose enters the cell through a PEP:phosphotransferase system that phosphorylates the galactose residue of lactose through a specific enzyme II. The phosphorylated lactose is then cleaved to form glucose and galactose-6-phosphate by a phospho-β-galactosidase activity. Galactose-6-phosphate is further catabolized through the tagatose pathway as described above.

Maltose, a disaccharide of glucose, can be utilized by several bacteria (e.g., *E. coli*, *Enterococcus faecalis*) and fungi (e.g., *S. cerevisiae*). In *E. coli*, genes for the uptake of maltose and maltodextrins (*malEGF*, *malK*, and *lamB*) and their degradation (amylomaltase and maltodextrin phosphorylase; *malPG* operon) are induced by maltose/maltodextrins. Maltodextrin can either be acted on by amylomaltase, which releases a D-glucose that can be converted to glucose-6-phosphate by HK, or maltodextrin phosphorylase, which releases glucose-1-phosphate that can be converted to glucose-6-phosphate by phosphoglucomutase. Maltose can be cleaved by β-glucosidase to yield two glucose molecules.

Melibiose (α-D-galactose-(1 → 6)-D-glucose) and/or raffinose (α-D-galactose-(1 → 6)-D-glucose-(1 → 2)-β-D-fructose) can be catabolized by several species of enteric bacteria (e.g., *E. coli*, *Salmonella*, and *Bacteroides*). Both *E. coli* and *Salmonella* are able to transport melibiose into the cell via a cation (sodium)-sugar cotransport system and degrade it to galactose and glucose by producing an α-galactosidase. *Bacteroides ovatus* also produces an α-galactosidase that allows for growth on both melibiose and raffinose.

Trehalose is a disaccharide of glucose linked through an α-1,1 linkage. In *E. coli* and *Salmonella*, trehalose is transported into the cell through the PEP-phosphotransferase system using a specific enzyme II activity to form trehalose-6-phosphate. Trehalose-6-phosphate is then hydrolyzed by trehalase to glucose and glucose-6-phosphate for utilization via the EMP pathway.

Sucrose or α-D-glucopyranosyl-($1 \leftrightarrow 2$)-β-D-fructofuranoside is a disaccharide composed of glucose and fructose. Sucrose can be utilized by a variety of bacteria (some strains of *E. coli*, *Clostridium*, *Bifidobacterium*) and yeasts (e.g., *S. cerevisiae*). In *E. coli* and *Clostridium*, sucrose is transported into the cell via the PEP:phosphotransferase system using a specific enzyme II activity to phosphorylate sucrose to form 6-phosphoglucose-fructose. The 6-phosphoglucose-fructose is then hydrolyzed by a fructosidase activity to glucose-6-phosphate and fructose, which can then enter into the EMP pathway.

Monosaccharide utilizations

Several monosaccharides other than glucose can be utilized by a variety of bacteria to varying degrees. However, in all cases they are typically converted into some intermediate that allows for entry into one or more of the central glycolytic pathways discussed previously.

As discussed above, the hexose galactose, released from lactose degradation, can be utilized by cells through either the tagatose pathway or the Leloir pathway depending on the organism. However, to grow on exogenous galactose as a carbon-energy source the cell must first get it into the cytoplasm. This is typically accomplished by active transport. In *E. coli* and *Salmonella*, D-galactose is transported via a pmf-driven galactose permease (encoded by *galP*); for *E. coli* galactose can enter through the β-methylgalactoside permease or Mgl system (encoded by *mglA*, *mglB*, and *mglC*). Once inside the cell the galactose is catabolized via the Leloir and EMP pathways as described above.

D-Fructose, D-mannose, and L-sorbose are hexoses that can also be used as carbon-energy sources by a variety of microbes. In strains of *E. coli*, *Salmonella*, and other enterobacteria capable of utilizing these sugars, they are all transported into the cell through the PEP: phosphotransferase system. Each utilizes a sugar-specific enzyme II to phosphorylate the sugar. Fructose-1-phosphate is then phosphorylated by an ATP-dependent 1-phosphofurctokinase (encoded by the *fruK* gene in *E. coli* and *Salmonella*) to FBP for entry into the EMP pathway. Mannose-6-phosphate gets converted to fructose-6-phosphate by mannose-6-phosphate isomerase (encoded by the *manI* gene in *E. coli* and *Salmonella*); Fru-6P then enters into the EMP pathway. L-sorbose-1-phosphate is reduced by NAD(P)H/H$^+$ to glucitol-6-phosphate by NAD(P)H-linked sorbose-1-phosphate reductase (encoded by the *sorB* gene in *E. coli* and *Salmonella*). Glucitol-6-phosphate is then oxidized using NAD$^+$ by the enzyme glucitol-6-phosphate dehydrogenase to form fructose-6-phosphate, which then enters the EMP pathway.

In addition to hexoses, many microbes are able to utilize amino sugars such as *N*-acetyl-D-glucosamine (NAG) or D-glucosamine. Both these amino sugars are common components of polysaccharides present in microbial cell walls and on the surface of cells. In *E. coli* and *Salmonella*, both amino sugars can enter the cell via the PEP:phosphotransferase system. *N*-acetyl-D-glucosamine transport involves phosphorylation by either a specific enzyme II or the major glucose enzyme II activity. In comparison, D-glucosamine is phosphorylated by either the enzyme II for glucose or D-mannose. NAG-6-phosphate can then be used directly for peptidoglycan synthesis or can be deacetylated by a NAG-6-phosphate deactylase (encoded by *nagA* in *E. coli*) to D-glucosamine-6-phosphate (also formed during the transport of D-glucosamine); D-glucosamine-6-phosphate is converted to Fru-6P, which then enters the EMP pathway.

Many microbes to varying degrees can also utilize pentoses (e.g., L-arabinose, D-xylose, and D-ribose), or their sugar alcohol derivatives (e.g., D-arabitol, xylitol, and ribitol) and methylpentoses (e.g., L-fucose and L-rhamnose) as alternative carbon-energy sources. In *E. coli* and *Salmonella*, each is transported into the cell by at least one specific permease; L-arabinose and D-xylose can enter through either a high-affinity or a low-affinity transport system. L-Arabinose inside the cell is converted to L-ribulose by L-arabinose isomerase (encoded by *araA* in *E. coli*). L-Ribulose is then phosphorylated forming L-ribulose-5-phosphate, by L-ribulose kinase (encoded by *araB* in *E. coli*) using ATP. Similarly, D-xylose inside the cell is converted to D-xylulose by D-xylose isomerase (encoded by *xylA* in *E. coli*). D-Xylulose is then phosphorylated forming D-xylulose-5-phosphate by D-xylulose kinase (encoded by *xylB* in *E. coli*) using ATP. D-Ribose is phosphorylated by D-ribose kinase (encoded by *rbsK* in *E. coli*) using ATP forming D-ribose-5-phosphate, which is converted to D-ribulose-5-phosphate by D-ribose-5-phosphate isomerase (encoded by the *rpi* gene in *E. coli*). D-Arabitol and ribitol in the cytoplasm get oxidized by NAD$^+$ to D-xylulose and D-ribulose by D-arabitol dehydrogenase (encoded by the *atlD* gene in *E. coli*) and ribitol dehydrogenase (encoded by the *rtlD* gene in *E. coli*), respectively. The D-xylulose and D-ribulose are then phosphorylated to D-xylulose-5-phosphate and D-ribulose-5-phosphate using ATP through the actions of D-xylulose kinase and D-ribulose kinase, respectively. The D-xylulose-5-phosphate and D-ribulose-5-phosphate formed in the above pathways are then able to enter the PPP for further catabolism. The utilization of the methylpentoses, L-fucose, and L-rhamnose, is a little more detailed. Both enter the cell via specific permease systems (encoded by the *fucP* and *rhaP* genes, respectively, in *E. coli*). Once inside the cell both undergo isomerization by a specific isomerase activity (encoded by the *fucI* and *rhaA* genes, respectively, in *E. coli*) forming

L-fuculose and L-rhamnulose, respectively. The L-fuculose and L-rhamnulose are then phosphorylated to L-fuculose-1-phosphate and L-rhamnulose-1-phosphate using ATP via the actions of L-fuculose kinase and L-rhamnulose kinase activities (encoded by the *fucK* and *rhaB* genes, respectively, in *E. coli*), respectively. L-Fuculose-1-phosphate and L-rhamnulose-1-phosphate can then both undergo cleavage catalyzed by a specific aldolase activity to form DHAP and lactaldehyde. The latter can be converted to L-lactate and then pyruvate through successive dehydrogenase-mediated reactions. DHAP can then enter the EMP pathway to form another pyruvate, which can undergo further catabolism as discussed previously.

Glycerol (a sugar alcohol) and glycerol-3-phosphate are also potential carbon-energy sources that can be derived from phospholipids present in membranes. Glycerol enters the cell through facilitated transport; glycerol-3-phosphate enters via a permease. Once inside the cell, glycerol gets phosphorylated by glycerol kinase (encoded by the *glpK* gene in *E. coli*) using ATP. Glycerol-3-phosphate can then be used directly in biosynthesis or can be converted to DHAP by one of three possible enzyme activities, an aerobic or anaerobic glycerol-3-phosphate dehydrogenase (encoded by *glpD* and *glpAB*, respectively, in *E. coli*) or glycerol-3-phosphate oxidoreductase (encoded by *gpsA* in *E. coli*), depending on the environmental conditions. The DHAP can then enter into glycolysis.

Precursor Metabolites for Biosynthesis/Anabolism

In addition to their function in the generation of energy and O–R cofactors, the central metabolic pathways provide essential precursor metabolites for anabolism. The 13 precursor metabolites are needed for the *de novo* biosynthesis of amino acids, fatty acids, nucleotides, coenzymes/vitamins/cofactors, and other molecules. These are in turn required for the biosynthesis of DNA, RNA, proteins, and (phospho-)lipids for membranes as well as other cell structures. Some of these metabolites can be generated from multiple central metabolic pathways depending on the microorganism. However, the EMP pathway, PPP, and the TCA cycle are the most common pathways from which these precursors are produced (**Figure 10**).

Precursors Metabolites Derived from the EMP Pathway

Precursors metabolites derived from the EMP pathway (**Figure 2**) include glucose-6-phosphate (Glc-6P), fructose-6-phosphate (Fru-6P), dihydroxyacetone phosphate (DHAP)/glyceraldhyde-3-phosphate (GAP) (considered together here due to isomerization), 3-phosphoglycerate (3-PG), PEP, and pyruvate (**Figure 10**). Glc-6P can be converted to glucose-1-phosphate (Glc-1P) by phosphoglucomutase; Glc-1P is needed for the generation of intermediates for the biosynthesis of several polysaccharides present in microorganisms including glycogen, *O*-polysaccharide of Gram-negative bacterial LPS, and cell-wall polysaccharides of many fungi. Fru-6P can enter the PPP (**Figure 3**) for the synthesis of key precursor metabolites (discussed below). Fru-6P can also be converted to glucosamine-6-phosphate (GluNH$_3$-6P) by the GluNH$_3$-6P synthase or to other sugar units (e.g., mannose or fucose), which are needed for the biogenesis of cell structures such as the peptidoglycan of bacteria, cell walls of fungi, and surface glycolipids or glycoproteins of many microbes. DHAP and GAP are formed from the splitting of FBP in the EMP pathway; DHAP is also formed from the isomerization of GAP from the EMP pathway (also ED or PPK pathways; **Figures 4** and **5**). DHAP can be converted to glycerol-3-phosphate, which is needed for the *de novo* biosynthesis of membrane phospholipids and certain teichoic acids of some Gram-positive bacteria. GAP (along with pyruvate) is required for the nonmevalonate pathway of *de novo* isoprenoid biosynthesis present in most Gram-negative bacteria as well as *Bacillus* (*subtilis*), *Mycobacterium* (*tuberculosis*), *Chlamydia* (*trachomatis*), and a few others. Polyisoprenoids are needed for the biosynthesis of many membrane-associated compounds such as quinones (e.g., ubiquinone and menaquinone). The 3-phosphoglycerate formed in the first ATP-generating step of the EMP pathway (**Figure 2**) is a precursor of *de novo* serine biosynthesis; serine in turn is a precursor of *de novo* biosynthesis of glycine, cysteine, and tryptophan. Glycine functions in the biosynthesis of porphyrins and purines. PEP is a precursor in the biosynthesis of shikimate (an intermediate in chorismate biosynthesis) and chorismate. Chorismate is a key intermediate in the biosynthesis of the aromatic amino acids tyrosine, phenylalanine, and tryptophan (with serine and 5-phospho-D-ribosyl-1-pyrophosphate or PRPP) as well as quinones. Pyruvate, formed in the second ATP-generating step of the EMP pathway (**Figure 2**), not only serves as a precursor of acetyl-CoA and OAA production but also serves as a precursor for the *de novo* biosynthesis of the amino acids alanine, valine, leucine (with acetyl-CoA), isoleucine (with threonine), and lysine (with aspartate) as well as isoprenoid biosynthesis (with GAP; discussed above).

Precursors Metabolites Derived from the TCA Cycle

The TCA cycle is an important source of four precursor metabolites, acetyl-CoA, α-ketoglutarate, succinyl-CoA,

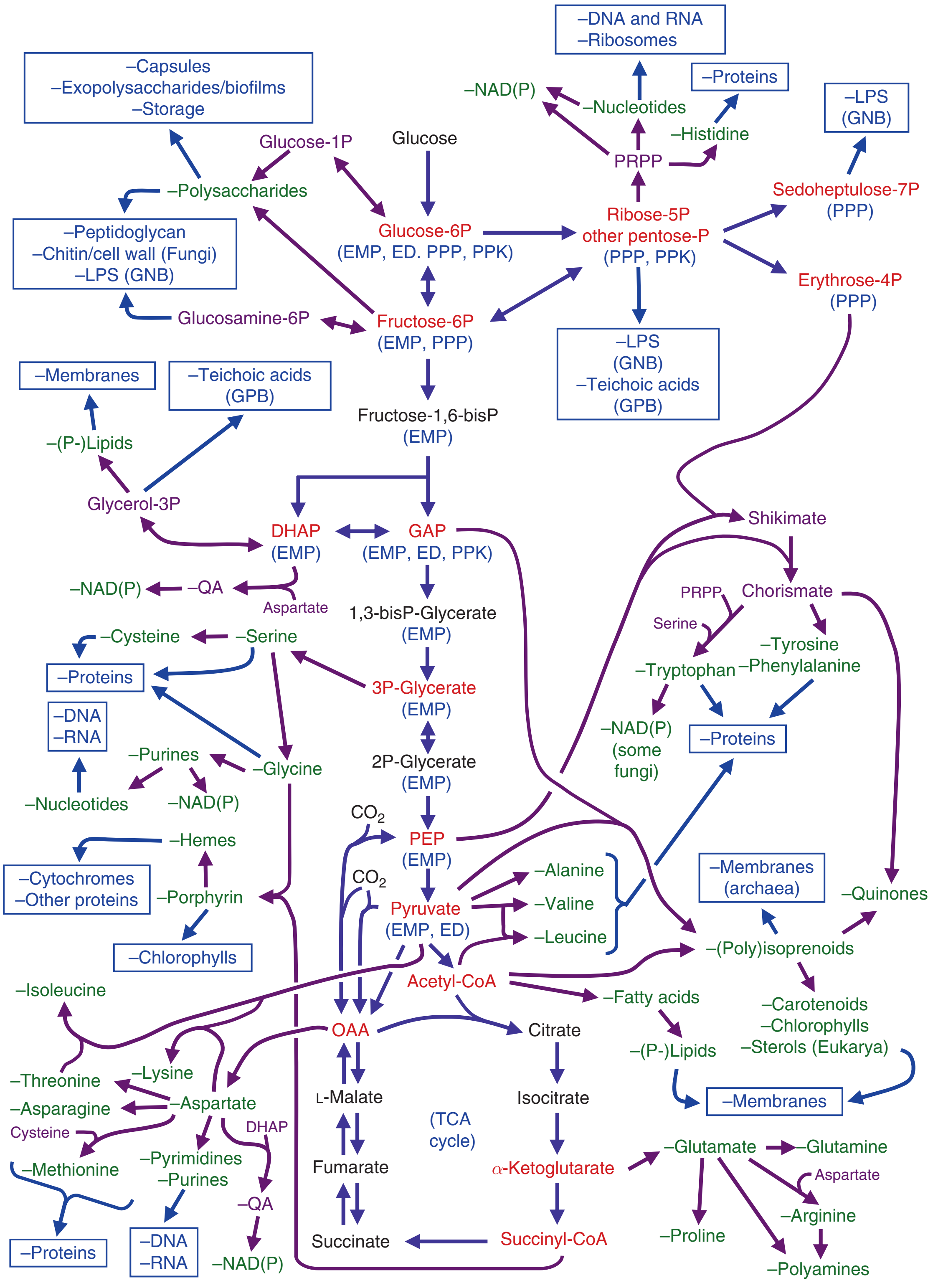
–DNA and RNA
–Ribosomes
–Capsules
–Exopolysaccharides/biofilms
–Storage
–NAD(P)
–Nucleotides
–Proteins
–LPS
(GNB)
–Histidine
Glucose-1P
Glucose
PRPP
Sedoheptulose-7P
(PPP)
–Polysaccharides
Ribose-5P
other pentose-P
(PPP, PPK)
–Peptidoglycan
–Chitin/cell wall (Fungi)
–LPS (GNB)
Glucose-6P
(EMP, ED. PPP, PPK)
Erythrose-4P
(PPP)
Glucosamine-6P
Fructose-6P
(EMP, PPP)
–LPS
(GNB)
–Teichoic acids
(GPB)
–Membranes
–Teichoic acids
(GPB)
Fructose-1,6-bisP
(EMP)
–(P-)Lipids
Glycerol-3P
Shikimate
DHAP
(EMP)
GAP
(EMP, ED, PPK)
–NAD(P)
–QA
Aspartate
PRPP
Chorismate
1,3-bisP-Glycerate
(EMP)
Serine
–Cysteine
–Serine
–Tyrosine
–Tryptophan
–Phenylalanine
–Proteins
3P-Glycerate
(EMP)
–DNA
–RNA
–NAD(P)
(some
fungi)
–Proteins
–Purines
–Glycine
2P-Glycerate
(EMP)
–Nucleotides
–NAD(P)
CO_2
–Hemes
PEP
(EMP)
–Membranes
(archaea)
–Cytochromes
–Other proteins
–Porphyrin
CO_2
–Alanine
–Quinones
–Valine
Pyruvate
(EMP, ED)
–Leucine
–Chlorophylls
–(Poly)isoprenoids
–Isoleucine
Acetyl-CoA
–Fatty acids
–Carotenoids
–Chlorophylls
–Sterols (Eukarya)
OAA
Citrate
–(P-)Lipids
–Threonine
–Lysine
–Aspartate
L-Malate
Isocitrate
–Membranes
–Asparagine
Cysteine
DHAP
(TCA
cycle)
–Methionine
–Pyrimidines
–Purines
Fumarate
α-Ketoglutarate
–Glutamate
–Glutamine
Aspartate
–QA
–Arginine
–Proteins
–DNA
–RNA
–NAD(P)
Succinate
Succinyl-CoA
–Proline
–Polyamines

Figure 10 General overview of the interconnection of central metabolism with biosynthetic/anabolic pathways. Intermediates of central metabolic pathways functioning as precursors in biosynthetic pathways are shown in red. Darker blue arrows indicate reactions of central metabolism or gluconeogenesis; purple arrows represent biosynthetic pathways; and lighter blue arrows indicate biogenesis of indicated cell structure/macromolecule or protein synthesis. ED = Entner–Doudoroff pathway; EMP, Embden–Meyerhof–Parnas pathway; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; LPS, lipopolysaccharide; NAD, nicotinamide adenine dinucleotide; PPK, pentose phosphoketolase pathway; PPP, pentose phosphate pathway; PRPP, 5-phospho-D-ribosyl-1-pyrophosphate; QA, quinolinic acid.

and OAA (**Figure 10**). Acetyl-CoA is included here since its condensation with OAA is the initiating step in the cycle. Acetyl-CoA has a variety of roles in both catabolic reactions (described earlier) and anabolic reactions. It is a key precursor in the biosynthesis of fatty acids for the production of phospholipids for membrane biogenesis; in addition, it is a precursor in the *de novo* leucine and mevalonate pathway of (poly)isoprenoid biosynthesis present in some bacteria (e.g., *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Lactobacillus*, *Myxococcus*), archaea (e.g., *Archaeoglobus*, *Pyrococcus*, *Methanococcus*, *Methanobacterium*), and eukaryotic microbes. In the Archaea, polyisoprenoids are a predominant component of the membrane, replacing the typical fatty acids present in bacterial and eukaryotic membranes. The intermediate α-ketoglutarate can be converted to glutamate by NADPH-dependent glutamate synthase or NAD(P)H-dependent (ammonia assimilating) glutamate dehydrogenase, which in turn can be converted to glutamine, arginine (with aspartate), proline, and various polyamines. Not shown in **Figure 10** are the major roles that glutamate and glutamine play in numerous amino group transfer reactions occurring in a variety of biosynthetic pathways in the cell. Succinyl-CoA is a precursor metabolite involved in *de novo* porphyrin biosynthesis (with glycine); porphyrins are intermediates for the synthesis of heme groups and chlorophylls in photosynthetic microorganisms. **Figure 10** does not show the additional role of succinyl-CoA in lysine and methionine biosynthesis (discussed below). Oxaloacetic acid is converted to aspartate by aspartate aminotransferase using glutamate as the amino group donor. Aspartate is a central intermediate in the *de novo* biosynthesis of lysine (with pyruvate and succinyl-CoA; except in yeasts and molds), threonine, asparagine, methionine (with cysteine and succinyl-CoA), purines and pyrimidines (needed for DNA and RNA), and quinolinic acid (an intermediate in *de novo* NAD biosynthesis).

Precursor Metabolites Derived from the PPP

The PPP is a source of the remaining three precursor metabolites: ribose-5-phosphate (may also be produced from intermediates in the PPK pathway), sedoheptulose-7-phosphate, and erythrose-4-phosphate. Ribose-5-phosphate plus ATP can be converted to PRPP and AMP by PRPP synthetase. PRPP supplies the ribose-5-phosphate moiety required in the *de novo* biosynthesis of purine and pyrimidine nucleotides as well as the pyridine nucleotide of NAD. PRPP is also essential for *de novo* histidine and tryptophan biosynthesis. Sedoheptulose-7-phosphate is a precursor needed for the *de novo* biosynthesis of L-glycero-D-mannoheptose and 2-keto-3-deoxyoctonate (with PEP; KDO) involved in LPS biosynthesis in Gram-negative bacteria. Erythrose-4-phosphate (with PEP) is a precursor in the *de novo* biosynthesis of shikimate, which is an intermediate in the synthesis of chorismate (discussed above).

Not shown in **Figure 10** are precursor metabolites, which function in the biosynthesis of the many coenzymes/vitamins/cofactors (e.g., riboflavin, thiamine pyrophosphate, CoA, vitamin B_{12}, folates, pyridoxal phosphate, and biotin) that are essential to cellular metabolism. These molecules are for the most part derived from various purine nucleotides (e.g., GTP in the case of folates and riboflavin or ATP in the case of CoA), amino acids (e.g., glutamate in the case of folates and aspartate in the case of pantothenate and CoA) as well as other metabolic intermediates such as chorismate (precursor for *p*-aminobenzoic acid or PABA).

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Metagenomics

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Function-Based Metagenomic Analysis
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Glossary

16S rRNA gene Highly conserved RNA involved in polypeptide synthesis that is commonly used for making phylogenetic inferences.

BAC Bacterial artificial chromosome.

contig A set of overlapping sequences derived from a single template.

cosmids Vectors containing *cos* sites for phage packaging that are used to clone large DNA fragments.

environmental DNA Genetic material extracted directly from a microbial community.

environmental gene tag Short sequence obtained from metagenomic sequencing.

fosmid Large-insert cloning vector based on the F-plasmid of *Escherichia coli*.

genome The genetic complement of an organism.

metaproteome The collective protein complements of a microbial community.

microbiome The collection of microorganisms residing in a particular habitat.

mini scaffold A single paired-end read.

read The output of an individual DNA sequencing reaction.

scaffold Contigs linked by overlapping regions.

Abbreviations

BAC	Bacterial artificial chromosome
CTAB	hexadecyltrimethylammonium bromide
EGTs	environmental gene tags
METREX	Metabolite-regulated expression
SIGEX	Substrate-induced gene expression

Defining Statement

Metagenomics is the study of the collective genomes of the members of a microbial community. It involves cloning and analyzing the genomes without culturing the organisms in the community, thereby offering the opportunity to describe the planet's diverse microbial inhabitants, many of which cannot yet be cultured.

Introduction

Prokaryotes are the most physiologically diverse and metabolically versatile organisms on our planet. Bacteria vary in the ways that they forage for food, transduce energy, contend with competitors, and associate with allies. But the variations that we know are only the tip of the microbial iceberg. The vast majority of microorganisms have not been cultivated in the laboratory, and almost all of our knowledge of microbial life is based on organisms raised in pure culture. The variety of the rest of the uncultured microbial world is staggering and will expand our view of what is possible in biology.

The challenge that has frustrated microbiologists for decades is how to access the microorganisms that cannot be cultured in the laboratory. Many clever cultivation methods have been devised to expand the range of organisms that can be cultured, but knowledge of the uncultured world is slim, so it is difficult to use a process based on rational design to coax many of these organisms into culture. Metagenomics provides an additional set of tools to study uncultured species. This new field offers an approach to studying microbial communities as entire units, without cultivating individual members. Metagenomics entails extraction of DNA from a community so that all of the genomes of organisms in the community are pooled. These genomes are usually fragmented and cloned into an organism that can be cultured to create 'metagenomic libraries', and these libraries are then subjected to analysis based on DNA sequence or on functions conferred on the surrogate host by the metagenomic DNA. Although this field of microbiology is quite young, discoveries have already been made that challenge existing paradigms and made substantial contributions to biologists' quest to piece together the puzzle of life.

Building Metagenomic Libraries

DNA has been isolated from microbial communities inhabiting diverse environments. Early metagenomic projects focused on soil and sea water because of the richness of microbial species (e.g., 5000–40 000 species/g soil) as well as the abundance of biocatalysts and natural products known to be in these environments from culture-based studies. While soil has been most sampled for metagenomic libraries, aquatic sediments, biofilms, and industrial effluents have also been successfully tapped, often because of their unique physicochemistries, for various biological activities. Metagenomic libraries constructed from DNA extracted from animal-associated microbial communities have also been the source of a number of novel biocatalysts (i.e., hydrolases, laccases, and xylanases), antibiotic resistance genes, and inter/intraspecies communication molecules. See **Figure 1** for an overview of metagenomic library construction and screening.

Preparing Metagenomic DNA

The physical and chemical structure of each microbial community affects the quality, size, and amount of microbial DNA that can be extracted. Accessing planktonic communities requires equipment that is capable of handling large volumes of water to concentrate sufficient microbial biomass to obtain enough DNA to build libraries. Contaminating chemicals and enzymes often remain in the water, making it relatively easy to isolate DNA without abundant contaminants. In contrast, inorganic soil components, such as negatively and positively charged clay particles, and biochemical contaminants, such as humic acids and DNases, make DNA extraction from soils, and subsequent manipulation, challenging. The process for removing contaminants determines both the clonability and the size of the DNA because many of the processes that effectively remove contaminants that inhibit cloning also shear the DNA. Physical disassociation of microbes from the semisolid matrix, typically termed 'cell separation', can yield a cell pellet from which DNA, especially high molecular weight (>20 kb) DNA, can be obtained. Immobilization of cells in an agarose matrix further reduces DNA shear forces and, following electrophoresis, facilitates separation of high molecular weight DNA from humic acids and DNases. Various commercial kits can be used to extract DNA from soil and other semisolid matrices. Applying multiple extraction methods to a DNA sample can yield minimally contaminated DNA. For example, a FastDNA Spin (Qbiogene) preparation followed by a hexadecyltrimethylammonium bromide (CTAB) extraction yields high quality DNA. Due to shear forces, low molecular weight DNA is typically isolated, but the physically vigorous nature of some of these methods can facilitate lysis of encapsulated bacteria, spores, and other microbial structures that are resistant to more 'gentle' lysis methods, thereby providing access to a greater, more diverse proportion of the microbial community for cloning.

Cloning Vectors and Metagenomic Library Structure

The choice of cloning vector and strategy largely reflects the desired library structure (i.e., insert size and number of clones) and target activities sought. To obtain a function encoded by a single gene, small DNA fragments (<10 kb) can be obtained and cloned in *Escherichia coli* into standard cloning vectors (e.g., pUC derivatives, pBluescript SK(+), pTOPO-XL, and pCF430). Various enzymes, such as amidases, hydrolases, cellulases, and antibiotic resistance determinants, have been identified in functional screens of metagenomic libraries harboring inserts smaller than 10 kb. Conversely, to obtain targets encoded by multiple genes, large DNA fragments (>20 kb) must be cloned into fosmids, cosmids, or bacterial artificial chromosomes (BACs), all of which can stably maintain large DNA fragments. Two vectors, namely pCC1FOS and pWE15, have been used for cloning large DNA fragments from various microbial communities. The pCC1FOS vector has the advantage that, when in the appropriate host (e.g. *E. coli* Epi300), its copy number can be controlled by addition of arabinose in the medium to increase DNA yield. Microbial sensing signals, antibiotic resistance determinants, antibiosis, pigment production, and eukaryotic growth modulating factors have been identified from metagenomic libraries constructed with pCC1FOS. Additionally, the presence of considerable flanking DNA on fosmid or BAC clone inserts facilitates phylogenetic inference about the source of the fragment.

Community Complexity and Metagenomic Library Structure

The determination of target insert size, cloning vector, and minimum number of library clones is governed by the type of genes that are sought and the complexity of the microbial community. Shotgun sequencing is usually conducted on small-insert clones, whereas successful functional studies can be performed on small- and large-insert clones. Small-insert metagenomic libraries constructed in plasmids that stably maintain up to 10 kb of DNA require 3–20 times more clones compared to libraries constructed in fosmids (30–40 kb inserts) or BACs (up to 200 kb inserts) to obtain comparable coverage of the same microbial community.

Community coverage is possible only in relatively simple microbial communities like the acid mine drainage, which contains only about five members. This

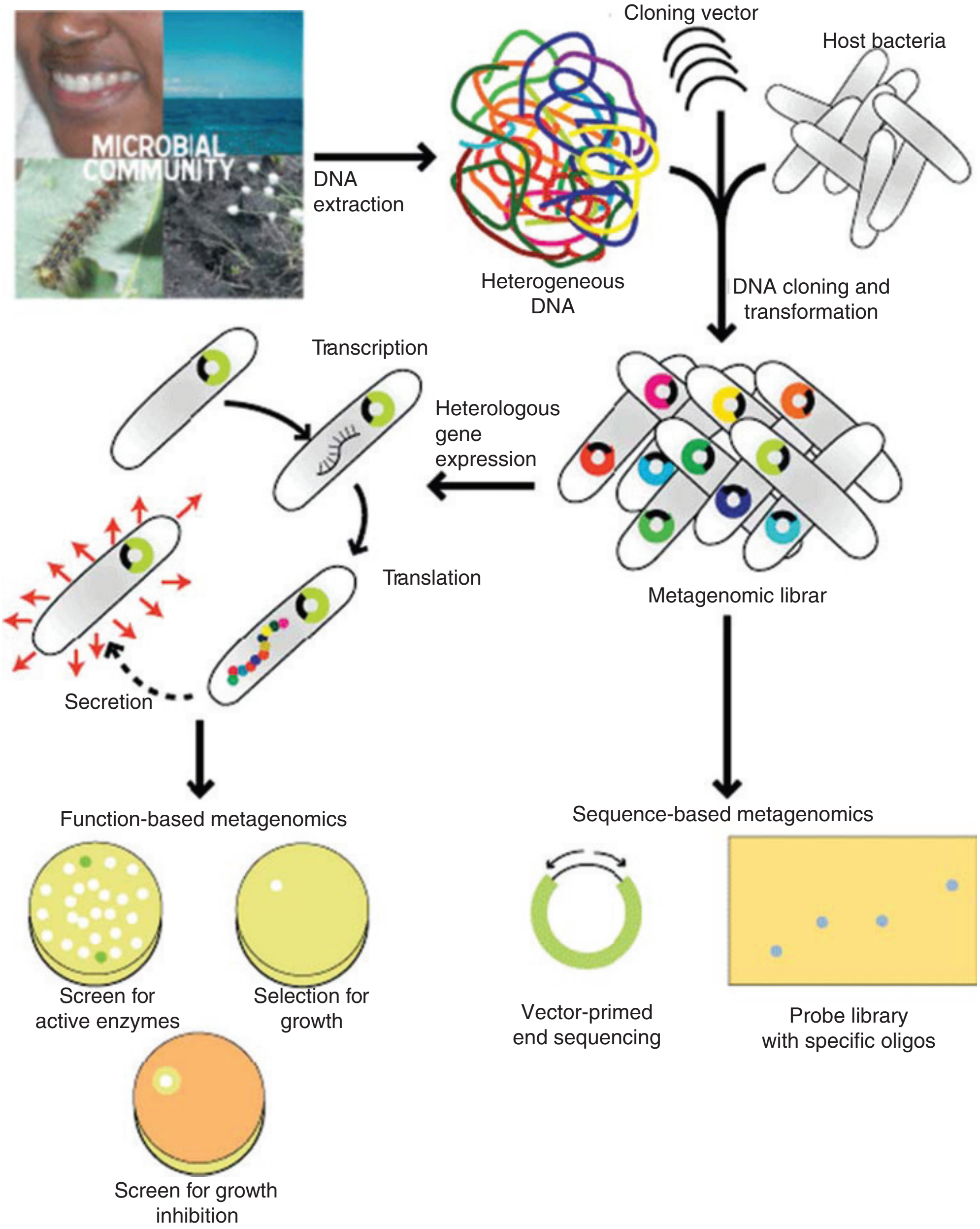
MICROBIAL COMMUNITY
DNA extraction
Heterogeneous DNA
Cloning vector
Host bacteria
DNA cloning and transformation
Metagenomic librar
Transcription
Heterologous gene expression
Translation
Secretion
Function-based metagenomics
Screen for active enzymes
Selection for growth
Screen for growth inhibition
Sequence-based metagenomics
Vector-primed end sequencing
Probe library with specific oligos

Figure 1 Metagenomics. Metagenomics is the study of the collective genomes of the members of a community. DNA is extracted directly from the community, cloned into a surrogate host, and then studied by sequencing or screening for expression of activities of interest. Many microbial communities have been tapped for metagenomic analyses. Following construction of a metagenomic library, two approaches can be taken to access the genomic information. Functional metagenomics requires that the host bacterium can express the recombinant DNA in either screens for active enzymes or antibiotic production or selections for growth under growth-suppressive conditions (e.g., nutrient deficiency or presence of an antibiotic). In sequence-based metagenomics, cloned DNA is randomly sequenced using vector-based primers or a specific gene is sought using complimentary oligonucleotides (oligos) to hybridize to arrayed metagenomic clones.

community was sampled and sequenced deeply with a high-density, large-insert metagenomic library, making it possible to reconstruct the genome of one member. In contrast, the metagenome of a very complex community such as that in soil can only be sampled, not exhaustively sequenced. With today's technology, a community can be sequenced to closure more quickly and cheaply with small- than large-insert clones, but future technology development may change this. In contrast, in activity-based analysis, large inserts are preferable because the probability that the target activity is encoded by any one clone is positively correlated with the size of the insert, and if the activity is encoded by a cluster of genes, they are more likely to be captured in a large insert.

Selecting and Transforming a Host Organism

Development of microorganisms to host metagenomic libraries has trended toward well-characterized and easily cultivated bacteria, with most work being conducted in *E. coli. E. coli* offers many useful tools for metagenomics, such as strains that harbor mutations that reduce recombination (*recA*) and DNA degradation (*endA*) and facilitate blue/white recombinant (*lacZ*) screening. Electroporation is the primary and most efficient method for introducing metagenomic DNA, especially large-insert libraries, into *E. coli.* Additionally, some vectors can be transferred from *E. coli* to other bacterial species by conjugation. This has been an important feature of activity-based analysis of metagenomic libraries. Libraries have been screened in *E. coli* with many successful outcomes, but the barriers to expression of genes from organisms that are distant from *E. coli* have led to screening of some libraries in other hosts. A number of clones containing interesting activities have been detected in only one host species and not in others. This suggests that the host background affects the expression of the genetic potential of the metagenomic library, and therefore the suitability of a particular host for the targeted screen must be considered. Sequence-based analysis of metagenomic libraries is conducted entirely in *E. coli.*

Storing Metagenomic Libraries

Library storage conditions should preserve clone viability as well as the original diversity of the library. *E. coli*-based metagenomic libraries can be prepared in liquid culture supplemented with vector-selective antibiotics and 10–15% glycerol and stored frozen in pooled aliquots. Pooled libraries are revived in fresh media supplemented with antibiotics, and brief incubation (1–2 h) at 30–37 °C on a rotary shaker is sufficient to initiate growth. Extended incubation could hinder recovery of the full metagenomic library due to overgrowth of some clones to the exclusion of others. The revived library is then suitable for screening or DNA preparation.

Sequence-Based Metagenomic Analysis

Sequence-based metagenomics is used to collect genomic information from microbes without culturing them. In contrast to functional screening, this approach relies on sequence analysis to provide the basis for predictions about function. Massive datasets are now catalogued in the 'Environmental Genomic Sequence' database, and each sequencing project is more informative than the last because of the accumulated data from diverse environments. As patterns emerge in the environmental sequences, sequence-based methods will be increasingly more informative about microbial communities.

Some studies use a gene of interest or 'anchor' to identify metagenomic clones of interest for further analysis. A metagenomic library is constructed and screened using PCR to amplify the anchor. Anchors are often a ribosomal RNA gene, but can also be a metabolic gene (e.g., a polyketide synthase). The clones that contain the anchor are then sequenced or further analyzed to provide information about the genomic context of the anchor. Thus, researchers can quickly focus on a clone of interest. For example, a marine picoplankton metagenomic library was screened by hybridization with a 16S rRNA gene probe and the 16S rRNA genes in the positive clones were then sequenced to provide a picture of the diversity of the community members.

Recently, the tremendous advances in DNA sequencing technology have made it feasible to sequence large libraries without preselection for clones containing a particular anchor. This has led to the accumulation of massive amounts of sequence data from uncultured microbes in several environments. Here we discuss a few examples of sequence-based metagenomic projects. These projects are also detailed in **Table 1**.

Anchor-Based Sequencing

Some early projects used rRNA or other genes as anchors to identify useful and informative clones from metagenomic libraries. For example, this technique was used to identify clones containing DNA from planktonic marine *Archaea.* Previously, 16S rRNA gene sequences had been recovered from several environmental samples, suggesting the presence of *Archaea* in nonextreme environments such as soil and open water, yet no cultured representatives of these clades were known. Stein and colleagues took a direct cloning approach to isolate genomic DNA from these organisms rather than attempting to culture them. They filtered seawater and prepared a fosmid library from the collected organisms. The library was probed to find small-subunit rRNA genes from archaeal species. One clone was found and completely sequenced. Several putative protein-encoding genes were identified, including some that had not yet been identified in *Archaea.*

Similarly, Béjà and colleagues constructed several BAC libraries from marine samples. These libraries were also screened by first probing for the presence of 16S rRNA genes. One 130-kb BAC clone was sequenced and found to contain a 16S rRNA gene from an uncultivated gamma-proteobacterium. Surprisingly, this clone contained a gene encoding a protein with similarity to rhodopsins, which are light-driven proton pumps found thus far only in Archaea and Eukarya. This new type of rhodopsin, called proteorhodopsin, suggests that bacteria may also use these proteins for phototrophy. This discovery highlights the

Table 1 Sequence-based metagenomics

Year	*Environment*	*Total amount sequenced*	*Number of reads*	*Vector*	*Assembly*	*Comments*	*Reference*
Anchor-based projects							
1996	Ocean	~10 kbp	36	pFOS1	Not attempted	Shotgun sequence clones form 38.5-kb cosmid insert	Stein *et al.* *Journal of Bacteriology* **178**: 591–599
2000	Ocean	128 kbp	Not reported	pIndigoBAC536	Entire BAC insert assembled	Found proteorhodopsin gene	Béjà *et al.* *Science* **289**: 1902–1906
2002	*Paederus* beetles	110 kbp	Not reported	pWEB	110 kbp assembled from several overlapping cosmids	Found putative pederin cluster is probably bacterial in origin	Piel *PNAS* **99**:14002–14007
Viral Metagenomics							
2002	Ocean	ND	~1934	pSMART	Contigs assembled at low stringency	Used specific techniques to enrich for viral DNA	Breitbart *et al.* *PNAS* **99**:14250–14255
Community Sequencing projects							
2004	Acid mine drainage	76.2 Mbp	103 462	pUC18	85% of reads in scaffold 2 kb or longer Combined length of 1183 scaffolds is 10.82 Mbp	Assembled two near-complete and three partial genomes	Tyson *et al.* *Nature* **428**: 37–43
2004	Sargasso Sea	1360 Mbp 265 Mbp	1 660 000 325 561	pBR322 derivative	64 398 scaffolds 217 015 miniscaffolds 215 038 singletons (data for Weatherbird only)	Larger Weatherbird Sample yielded assembly, smaller Sorcerer II did not	Venter *et al.* *Science* **304**: 66–74
2005	Minnesota soil Whale-fall	100 Mbp 3 × 25 Mbp	149 085 Not reported	Lambda ZAP	Analyzed without assembly for environmental gene tags and metaproteome	Assembly not possible for soil sample due to complexity	Tringe *et al.* *Science* **308**: 554–557
2006	Human gut	78 Mbp	139 521	pHOS2	14 572 scaffolds for 33 753 108 bp 40% of reads not assembled for an additional 44 Mbp	0.7X coverage of *B. longum* genome and 3.5X coverage of *M smithii* genome	Gill *et al.* *Science* **312**: 1355–1359

expansive potential and novelty of bacterial genes in the environment and further supports the hypothesis that much of microbial diversity remains undescribed.

Genes besides 16S rRNA genes can also be used as anchors. To identify the source of production of the antitumor compound pederin, Piel constructed a cosmid library from pederin-producing *Paederus* beetles and screened the library for pederin synthesis genes. A locus encoding the putative pederin synthesis genes was identified, and the locus seems to be bacterial in origin, suggesting symbiotic bacteria within *Paederus* beetles are the true producers of pederin. This analysis shows that metagenomics can be used to isolate genes of potential medical interest and confirms that it is the bacteria, not their insect hosts, that produce this antitumor compound.

Viral Metagenomics

The world is thought to contain 10^{32} uncharacterized viruses, and characterizing them by metagenomics may be more efficient and informative than finding a suitable host for each of them. Moreover, since most of their hosts have likely not been cultured, metagenomics represents the only way to access this viral diversity. Cloning viral genomes from an environmental sample faces additional challenges that are not necessarily barriers in metagenomics based on cellular organisms. For example, the viral DNA must be separated from abundant free DNA in the environment, including organismal DNA; viral genes that kill the host must be inactivated or they will prevent the cloning of the viral DNA; chemically modified viral DNA can be unclonable; and ssDNA and RNA viral genomes are not amenable to traditional cloning. Generally, researchers have purified intact virus particles by physical separation and then extracted the DNA from those particles. In one example, Breitbart and colleagues used a linker-amplified shotgun library method to analyze a viral metagenomes from the Pacific Ocean. They found that most viral sequences recovered were not highly similar to known viral sequences, suggesting that the global viral metagenome is still undersampled.

Community Metagenomics

The development of increasingly fast, accurate, and inexpensive sequencing technologies, coupled with significant improvements in bioinformatics, has made it feasible to conduct large-scale sequencing of DNA from multispecies communities. This development will advance our understanding of microbial diversity in nature. Freed from the constraint of cultivating microbes to access their genomes, researchers have accumulated vast quantities of microbial genomic information that could not have been gathered even a few years ago. Although whole genomes cannot currently be reassembled from shotgun sequencing of complex communities containing dozens, hundreds, or thousands of species, rapid advances in technology development make it likely that such feats are not far off.

The first environment to be the subject of a comprehensive sequence-based metagenomic effort was the acid mine drainage environment at Iron Mountain, California. Acid mine drainage results when pyrite dissolution facilitates microbial iron oxidation, which is accompanied by acid production. The simplicity of the acid mine drainage microbial community led to the assembly of five nearly complete or partial genomes without first separation of cells or cultivation of community members.

Approximately 76 million base pairs (Mbp) were sequenced from small-insert libraries from the acid mine drainage biofilm. The community consisted of three bacterial and three archaeal lineages, thus raising the possibility of genome reassembly for many if not all of the members. To achieve this, the sequences were first assembled into scaffolds. Then the scaffolds were sorted into ‘bins’ based on their G+C content. Binning is the first step in assigning the scaffolds to a unique genome. The scaffolds in each bin were then sorted based on the degree of coverage, assuming that a more abundant member of the community would contribute more DNA to the library, and therefore be more highly represented in the sequence data and that all genes within one organism’s genome should be similarly represented. Thus, the high G + C bin was separated into a 10X coverage genome and a 3X coverage genome. These two genomes were found to represent a *Leptospirillum* group II genome and a *Leptospirillum* group III genome, respectively. A nearly complete, 2.23-Mb genome of the group II genome was assembled. Similarly, the 10X coverage genome in the low G + C bin represented a nearly complete genome of the archaeon *Ferroplasma* type II. Partial genomes were also identified for a *Ferroplasma* type I strain and a ‘G-plasma’ strain.

In addition to providing a model for genome reassembly, the acid mine drainage study led to tremendous insight into the habitat and physiology of the community members. The representation of various functions was recorded, and a chemical model for the flow of energy, nutrients, and electrons was developed and tested.

Another pivotal study in sequence-based metagenomics involved the large-scale sequencing of libraries constructed from the DNA of microbes living in the Sargasso Sea. More than one billion base pairs of DNA were sequenced, representing approximately 1800 genome equivalents. Clones were end-sequenced to provide paired-end reads and assembled into scaffolds, mini scaffolds (with a single paired-end read), or left as single reads. Again, the sequences were sorted into bins, based on depth of coverage, oligonucleotide frequencies, and similarity to known genomes. From these data, several nearly complete genomes were assembled, as well as ten megaplasmids. Sequence analysis predicted that 1 214 207 novel proteins were encoded in the environmental DNA. The analysis highlighted the presence of related strains of a given species in the sample, raising the complicating factor of strain heterogeneity in metagenome analysis.

When the community is sufficiently complex to prevent reassembly of genomes, other strategies must be undertaken to find patterns. For example, one study compared four metagenomic libraries, one from soil and three from oceanic 'whale-fall' samples (decomposing whale carcasses on the ocean floor), all of which were too complex for genome reassembly or even construction of contigs or scaffolds. Using paired-end sequences from small-insert libraries, 'environmental gene tags' (EGTs) were identified, 90% of which contained predicted genes, thus allowing for a global predicted metaproteome analysis without assembly of the DNA into larger scaffolds. This approach may be useful to find patterns in predicted protein functions and metabolic properties in many different environments.

In a powerful study, sequence-based metagenomics was used to investigate the microbiome of the human ecosystem. Since many of the microorganisms living in the human gut have not yet been cultured, metagenomics allows a direct approach to this microbial community. The data revealed nearly complete genomes of strains of *Bifidobacterium longum* and *Methanobrevibacter smithii*, an archaeon frequently found in the gut ecosystem. Gut microbes contribute many functions to human metabolism, including glycan degradation and fatty acid synthesis, and a number of these functions were identified and their diversity assessed by metagenomics. Scale-up of this approach could yield near-complete genomes of many of the important microbes in our own gut. Comparative studies among individuals, during infant development and after antibiotic ingestion, and among people consuming different diets are beginning to reveal both the idiosyncratic nature of each person's gut microbiota, which may provide a signature as unique as a fingerprint, and the microbial motifs that define health and disease.

Sequence-based metagenomics has the potential to revolutionize our understanding of microbial diversity and function on earth. However, even these initial studies have raised several questions of methods and technology. It is apparent that further advances in bioinformatics are needed to handle the vast quantities of data derived from these projects. In addition, the species richness of the communities, coupled with the genetic complexity of populations of each species, necessitates sequencing extremely large libraries to approach complete coverage. Finally, uneven species distribution, leading to the overrepresentation of abundant genomes in libraries, makes the desired library size even larger in order to capture rare species. The acceleration of sequencing, techniques that remove the most abundant sequences, and computational tools will enhance sequence-based metagenomic analysis.

Function-Based Metagenomic Analysis

Functional metagenomics involves identification of clones that express activities conferred by the metagenomic DNA. Sequence-based metagenomics has revealed physiological and ecological capacity that extends well beyond that of the culturable minority. Activity-based metagenomics provides an opportunity to circumvent culturing and to survey a community's functions (**Table 2**). Function-based metagenomics, unlike sequence-driven approaches, does not require that genes have homology to genes of known function, and it offers the opportunity to add functional information to the nucleic acid and protein databases.

Screening Metagenomic Libraries for Novel Enzymes

Microorganisms have always been a prime source of industrial and biotechnological innovations, but until recently applications have been derived from cultured organisms. Metagenomics presents the possibility of discovering novel biocatalysts from microbial communities that either confounded cultivation or failed to yield new culture isolates upon repeated attempts. Assays that have historically been used to identify enzymes (e.g., amylases, cellulases, chitinases, and lipases) in cultured isolates have been applied successfully to functional metagenomics. Function-based metagenomic analysis of Wisconsin agricultural soil yielded 41 clones having either antibiotic, lipase, DNase, amylase, or hemolytic activities among BAC libraries containing 28 000 clones with an average insert size of 43 kb. The frequency of finding active clones in these libraries ranged from 1:456 to 1:3648, which is similar to the results from other metagenomic surveys for biocatalysts, thereby highlighting the need for robust assays for functional analysis.

Exploiting Environmental Physicochemical Conditions for Biocatalysts Discovery

One approach intended to increase the likelihood of finding certain activities is to build metagenomic libraries from environments that are enriched for bacteria with the desired function. For example, a search for cellulases focused on the liquor of an anaerobic, thermophilic, lignocellulosic digester. Four clones expressing cellulolytic activity were identified, all of which had activity optima at pH 6–7 and 60–65 °C – conditions similar to those in the digester.

Table 2 Functional metagenomics surveys

Target gene	*Source*	*Host strain*	*Cloning vector*	*Insert size (kb)*	*Number of clones*	*Active clones*	*References*	*Extraction method*
Cellulases	Feedstock	*E. coli*	N.R.	N.R.	N.R.	4	Healy *et al.* *Applied Microbiology and Biotechnology* 43: 667–674	Direct
Biocatalysts and Antimicrobials	Soil	*E. coli* DH10B	pBeloBAC11	27/44.5	3646/ 24 546	41	Rondon *et al.* *Applied and Environmental Microbiology* 66: 2541–2547	Direct
Tetracycline resistance determinants	Oral cavity	*E. coli* TOP10	pTOPO-XL	0.8–3	450	1	Diaz-Torres *et al.* *Antimicrobial Agents and Chemotherapy* 47: 1430–1432	Direct
Xylanases	Insect	*E. coli*	Lambda ZAP	3–6	1 000 000	4	Brennan *et al.* *Applied and Environmental Microbiology* 70: 3609–3617	Direct
Amidases	Soil	*E. coli* TOP10	pZerO-2	5.2	8000 / 25 000	5	Gabor *et al.* *Environmental Microbiology* 6: 948–958	Direct/ enrichment
Aminoglycoside resistance determinants	Soil	*E. coli* DH10B	pCF430/pJN105	1.9–65	1 186 200	10	Riesenfeld *et al.* *Environmental Microbiology* 6: 981–989	Direct
Signal molecules	Soil	*E. coli* Epi300	pCC1FOS/ pSuperBAC/ pCC1BAC	1–190	180 000	3	Williamson *et al.* *Applied and Environmental Microbiology* 71: 6335–63*44*	Direct
Catabolic enzymes	Aquatic	*E. coli* JM109	p18GFP	7	150 000	∼35	Uchiyama *et al.* *Nature Biotechnology* 23: 88–93	Direct
Antibiotic desistance determinants	Oral cavity	*E. coli* TOP10/ Epi300	TOPO-XL/ pCC1FOS	0.8–3/∼40	1260/600	90/14	Diaz-Torres et al. *FEMS Microbiology Letters* 258: 257–262	Direct
Signal molecules	Insect	*E. coli* DH10B	pBluescript II KS (+)	3.3	800 000	1	Guan *et al.* *Applied and Environmental Microbiology* 73: 3669–3676	Direct

Another study was predicated on the finding that wood- and plant-eating insects are rich sources of enzymes involved in degradation of complex carbon polymers such as cellulose and xylan. These polysaccharides are the primary nutrient source for both the insect and the microbial community it harbors, and therefore should enrich for species that produce glycosyl hydrolases. Small-insert libraries constructed from termite and lepidopteran gut microbial DNA (1×10^6 clones) contained four clones with xylanase activity. These clones harbored genes that encoded xylanase catalytic domains with low sequence similarity (33–40%) to other glycosyl hydrolases. Not surprisingly, the enzymes most closely related to three of the four xylanases identified in this study were found elsewhere in gut-associated microbes. Prior knowledge of the habitat, based on either culture-based studies or metagenomic sequence analysis, facilitates shrewd choices that match habitat with the function that is sought.

Artificial Enrichment for Biocatalyst Discovery

Just as the environment can compel microbial communities to retain members with specific biochemical abilities, microbial communities can be actively manipulated prior to metagenomic library construction to enrich for desired activities. One study focused on the discovery of amidases that convert D-phenylglycine amide derivatives into key intermediates for the production of semisynthetic β-lactam antibiotics. Soil was added to minimal medium that had either D-phenylglycine amide or a mixture of various amides as the sole nitrogen source. Libraries were constructed in a leucine-auxotrophic *E. coli* strain and screened on the medium containing only phenylacetyl-L-leucine or D-phenylglycine-L-leucine, either of which would select for the growth of clones capable of hydrolyzing the amide compounds. According to DGGE analyses, enrichment cultures showed 64–77% lower bacterial diversity than in the original sample before enrichment, suggesting that the enrichment conditions enhanced growth of those bacteria that could utilize the amides as a nitrogen source and limited growth of the rest of the community, thereby reducing the diversity of the community. Four amidase-positive clones were identified from metagenomic libraries constructed from enrichment cultures and all had low to moderate homology to known enzymes. Two amidase-positive clones had low homology to hypothetical proteins. Following extensive amide substrate profiling, a single clone (pS2) was found to catalyze the synthesis of penicillin G from 6-aminopenicillanic acid and phenylacetamide. The pS2-encoded enzyme facilitated accumulation of twofold higher maximum level of penicillin G than *E. coli* penicillin amidase and performed better in amoxicillin and ampicillin production experiments. These analyses show that activity-based screening of metagenomic libraries can yield unique biocatalysts of ecological relevance and biotechnological importance, and some of these may be superior to those found in cultured organisms.

Rapid Discovery of Novel Antibiotic Resistance Genes

Genes that confer antibiotic resistance to bacteria are of great public health, pharmacological, and biological importance. Lateral gene transfer and broad antibiotic usage have resulted in wide distribution of antibiotic resistance genes in microbial communities. As a result, many antibiotic treatments are rendered less effective or totally ineffective against pathogens (e.g., methicillin-resistant *Staphylococcus aureus*). Characterization of the resistance genes in uncultured organisms may identify resistance determinants that will appear in clinical settings in the future. Characterization of resistance in environments that have not been influenced directly by human use of antibiotics could point to the origins of certain resistance determinants. A comprehensive understanding of resistance mechanisms in culturable and uncultured bacteria will improve drug design, by providing clues to how resistance can be combated. Additionally, antibiotic resistance and synthesis genes usually cluster together in microbial genomes. Therefore, antibiotic resistance genes can provide a signpost for biosynthetic pathways residing in nearby DNA.

Antibiotic resistance provides technical advantages over most other characteristics studied with metagenomics. Because the frequency of active clones is low, there is a substantive advantage to using a selection in which only the desired clones survive, rather than a screen, in which all clones need to be addressed to determine whether they express the desired function. When antibiotic-resistant clones are the target, metagenomic libraries are cultured in the medium containing the appropriate antibiotic and only those clones that contain and express cognate resistance genes grow. Using this strategy, a number of studies have identified clones that carry resistance to antibiotics such as tetracycline or aminoglycosides such as kanamycin, tobramycin, and amikasin. In one study, 1 186 200 clones from small- and large-insert soil metagenomic libraries were selected for aminoglycoside resistance and ten surviving clones were identified. Some of these clones carry resistance genes that are similar to known aminoglycoside resistance genes, some carry resistance genes that are distantly related to known resistance genes, and some resistance genes do not have detectable similarity to any known resistance determinants. The results of the antibiotic resistance studies provide a model for the rest of the metagenomic field. Two lessons are evident. First, the uncultured world contains some genes that are quite familiar based on studies of cultured organisms as well as more exotic ones that represent new classes of genes or proteins for a known function. Second, selections provide the power to rapidly identify clones of interest, circumventing the tedious screening step that forms the basis – and bottleneck – of many function-driven metagenomic studies.

Intracellular Functional Screens

The daunting challenge of screening massive libraries with millions of members has been met with a novel type of screen, in addition to the selections described in the previous section. In an effort to devise screens that are both rapid and sensitive, the concept of 'intracellular' screens emerged in which the detector for the desired activity resides in the same cell as the metagenomic DNA. The first two intracellular screens, metabolite-regulated expression (METREX) and substrate-induced gene expression (SIGEX) (**Figure 2**), meet the criteria of speed and sensitivity and provide prototypes for sensitive screens that are capable of reporting poorly expressed gene products and can utilize technologies (e.g., fluorescence-activated cell sorting) that rapidly screen millions of clones.

METREX screening detects molecules that mimic quorum-sensing signal molecules (**Figure 2**(**a**)). These compounds stimulate transcription, regulated by the LuxR protein and initiated from the *luxI* promoter, which is linked to a reporter, such as the gene encoding green fluorescent protein. The typical quorum-sensing inducers are acylated homoserine lactones, but metagenomics has revealed other classes of molecules that can function similarly. Since the metagenomic DNA and the broad-specificity reporter plasmid are both present within the host cell, even low concentrations of poorly expressed metagenomic gene products can be detected. In one study, 180 000 clones from soil metagenomic libraries were subjected to the METREX screen; 11 clones that stimulated Gfp expression were identified. Additionally, two clones inhibited Gfp expression in the presence of 80 n mol l^{-1} *N*-(3-oxohexanoyl)-L-HSL, a typical quorum-sensing signal molecule isolated from cultured organisms, which binds to LuxR and stimulates transcription from the *luxI* promoter. Interestingly, only one of the Gfp-stimulating clones could be detected in an overlay-based Gfp-reporter screen, indicating that the intracellular screen detects clones that would be lost in a standard screen for quorum-sensing signal compounds in which the active molecule needs to diffuse out of the producing cell and into the cell containing the reporter. Most of these clones had no sequence similarity to genes known to direct production of quorum-sensing stimulating or inhibiting compounds.

SIGEX facilitates the rapid identification of promoters that drive transcription in the presence of a particular catabolite (**Figure 2**(**b**)). This can be used to identify pathways that are regulated by the metabolite. Degradative pathways are often regulated by the compound that is degraded, so SIGEX is of interest for rapid identification of new gene clusters for bioremediation. Of 152 000 clones from a metagenomic library derived from an aquatic microbial community, 33 clones were induced by benzoate and two by naphthalene. A wide variety of genes were resolved in these screens, reflecting not only the sensitivity of the assay but also the broad impact of aromatic hydrocarbons on bacterial community gene expression.

Further developments in high-throughput screening will enhance discovery in function-driven metagenomics just as advancements in sequencing and bioinformatics will accelerate discovery in sequence-driven metagenomics.

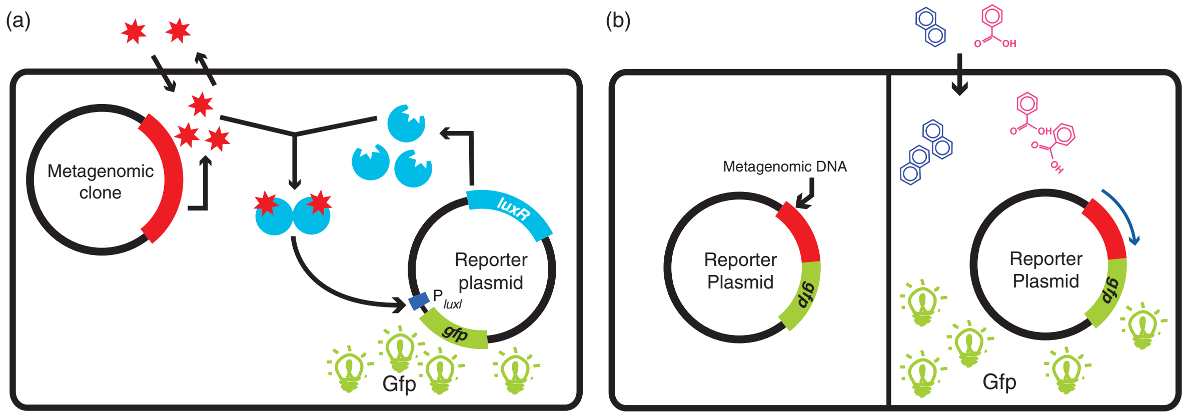

Figure 2 Intracellular screens for metagenomic libraries. (a) Metabolite-regulated expression (METREX): diffusible metagenomic gene products (red) stimulate dimerization of LuxR proteins (blue), which in turn induce expression of Gfp from the *luxI* promoter to produce light (green). Gene products interacting directly with the luxI promoter could also be detected. (b) Substrate-induced gene expression (SIGEX): Metagenomic DNA is cloned into a promoter-trap vector containing a promoterless Gfp-reporter downstream of the cloning site. Promoters in the metagenomic DNA that respond to specific catabolites induce expression of Gfp.

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Metal Extraction and Biomining

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Defining Statement
Microbial Transformations of Metals
Microorganisms that Solubilize Metals
Microbial Extraction of Metals from Ores and Biomining
Acidiophilic Microorganism–Mineral Interaction
Mechanisms Involved in Metal Solubilization by Acidophiles
Biomining in the Postgenomic Era
Environmental Effects of Metals Solubilization and Bioremediation
Conclusions
Further Reading

Glossary

biofilm A layer of adhesive exopolymeric substances attached to a surface and secreted by microorganisms forming colonies on it.

bioleaching Refers to the microbial conversion of an insoluble metal (usually a metal sulfide or oxide) into a soluble form (metal sulfate).

biomining The use of microorganisms to recover metals in industrial operations.

bioremediation Use of microorganisms to remove toxic chemicals from the environment.

biosorption Refers to the binding of metal ions by whole biomass (living or dead).

chemolithoautotroph A microorganism that fixes CO_2 and obtains its energy by the oxidation of inorganic compounds.

consortium A group of microorganisms living together and in which each individual benefits from the others.

genome The complete set of genes present in an organism.

genomics Refers to mapping, sequencing, and analyzing genomes.

proteome The total complement of proteins present in a cell at any one time.

proteomics Genome-wide study of the structure, function, and regulation of proteins in the cell.

systems microbiology Considers microorganisms or microbial communities as a whole to create an integrated picture of how a microbial cell or community operates.

Abbreviations

2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
AMD	acid mine drainage
DGGE	denaturing gradient gel electrophoresis
ESI-MS	electron spray ionization MS
FISH	fluorescence *in situ* hybridization
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometer
HPLC	High performance liquid chromatography
MS	mass spectrometry
QS	quorum sensing
SDO	sulfur dioxygenase
SOR	sulfite oxidoreductase
TEM	transmission electron microscopy

Defining Statement

Microorganisms interact with heavy metals, transforming them by uptake, bioaccumulation, bioprecipitation, bioreduction, biooxidation and other mechanisms. Some of these activities result in the solubilization or extraction of metals which are successfully used in industrial biomining processess to recover valuable metals or in biorremediation to remove toxic metals from contaminated soils.

Microbial Transformations of Metals

Microorganisms interact with metals by several mechanisms, most of which are shown in **Figure 1**. All bacteria require several metals that are essential for their functioning and the uptake of most of them is metabolism- or energy-dependent. For this bioaccumulation, they possess specific or general energy-dependent metal transporters to directly incorporate them or through chelation by

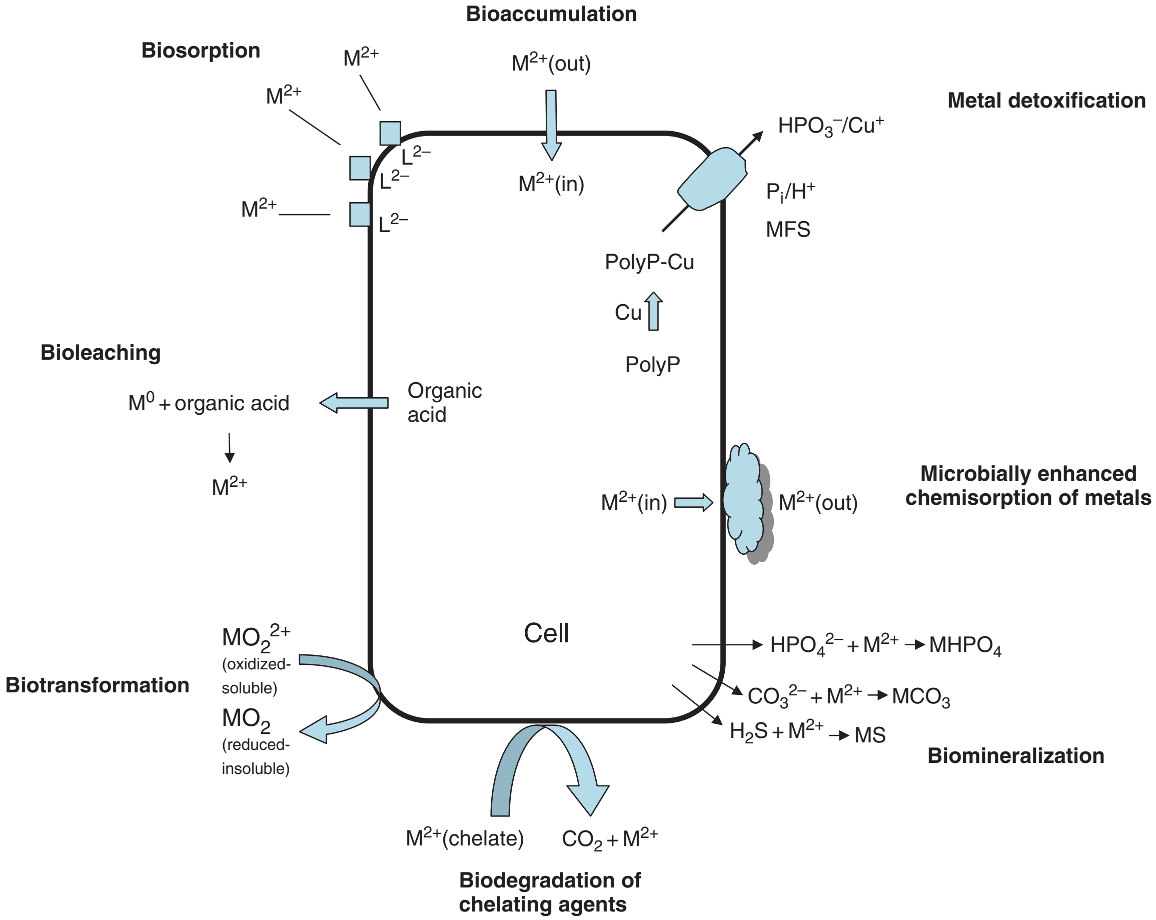
Bioaccumulation
Biosorption
M^{2+}
$M^{2+}(out)$
M^{2+}
Metal detoxification
HPO_3^-/Cu^+
L^{2-}
L^{2-}
$M^{2+}(in)$
P_i/H^+
M^{2+}
L^{2-}
MFS
PolyP-Cu
Cu
PolyP
Bioleaching
M^0 + organic acid
Organic acid
M^{2+}
Microbially enhanced chemisorption of metals
$M^{2+}(in)$
$M^{2+}(out)$
Cell
MO_2^{2+} (oxidized-soluble)
$HPO_4^{2-} + M^{2+} \rightarrow MHPO_4$
Biotransformation
$CO_3^{2-} + M^{2+} \rightarrow MCO_3$
MO_2 (reduced-insoluble)
$H_2S + M^{2+} \rightarrow MS$
Biomineralization
M^{2+}(chelate)
$CO_2 + M^{2+}$
Biodegradation of chelating agents

Figure 1 A general scheme showing the most common bacteria–metals interactions. Modified from Lloyd JR, Anderson RT, and Macaskie LE (2005) Bioremediation of metals and radionuclides. In: Atlas RM and Philp JC (eds.) *Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup*, pp. 293–317. Washington, DC: ASM Press.

means of organic compounds such as siderophores in the case of iron. Some of these transporters are used to bioaccumulate metals inside the cell. This can be done by sequestering the metal by proteins rich in cysteine or histidines or they can be chelated by inorganic polyphosphates (polyP), which are long chains of phosphate molecules joined through phosphodiester bonds and are highly negatively charged at neutral pH. Microorganisms such as *Acinetobacter* spp. bioprecipitate metals in the form of metal phosphates via hydrolysis of stored polyP depending upon alternating aerobic (polyP synthesis) and anaerobic (polyP hydrolysis and phosphate release) periods.

The metabolism-independent sorption of heavy metals or biosorption refers to the binding of metal ions by whole biomass (living or dead). It can take place by adsorption in which metals accumulate at the surface of the biomass and by absorption or a rather uniform penetration of the metal ions from a solution to another phase. Biomolecules present in the biomass contain several chemical groups that act as ligands (L^{2-} in **Figure 1**) for the biosorption of the metal ions. The most common are the amino, carboxyl, phosphate, and sulfhydryl groups present in proteins, nucleic acids, and polysaccharides.

Microorganisms can also catalyze several biotransformations. They transform most toxic metals into less soluble or less volatile forms. One example is the reduction of metals such as Cr(VI) to Cr(III), U(VI) to U(IV), Te (VI) to Te(0) and many others that results in the precipitation of the metal under physiological conditions. Another very well-studied biotransformation of a toxic metal is the bioreduction of Hg(II) to the relatively nontoxic volatile elemental Hg(0).

Microorganisms also precipitate metals in the form of carbonates, hydroxides, or insoluble sulfides and phosphates. This constitutes a biomineralization, and due to the very low solubility products of these compounds formed, most of the soluble metals would be removed by precipitation in their liquid medium (**Figure 1**).

Microorganisms that Solubilize Metals

A basic prerequisite for life is the existence of a chemical redox gradient to convert energy. This allows energy flow and its use in the biochemical reactions of the cells. Most cells use organic compounds like sugars as fuels (electron donors). Their oxidation in the presence of oxygen or respiration provides carbon to the cell generating as end products CO_2 and H_2O and energy in the form of electrons. This energy is stored mainly in the form of ATP to be used in the cell metabolism. A group of special microorganisms (Bacteria and Archaea) known as chemolithoautotrophs are capable of using minerals as fuels. Their oxidation generates electrons to obtain ATP and the carbon is obtained by fixing CO_2 from the air. During this aerobic mineral oxidation metals are solubilized or bioleached.

Anaerobic respiration can also solubilize metals. In this case, the mineral acts as an electron acceptor and the metal is solubilized under reducing conditions. Several microorganisms are capable of reducing heavy metals and couple this reduction with the oxidation of energy sources such as hydrogen or organic compounds (formate, lactate, amino acids, and others). Characteristic examples are the reduction of Fe and Mn, which account for good part of organic carbon turnover in several environments. Several of the dissimilatory metal-reducing bacteria not only reduce Fe and Mn but also other metals such as the toxic Cr(VI) and U(VI) and, therefore, microorganisms like *Shewanella oneidensis* could also be used for bioremediation.

The reactions just mentioned are part of normal biogeochemical processes in nature. They are performed under neutral conditions or as the majority of microorganisms do, at very high acidic values (pH 1–3 usually). This article will concentrate mainly on reviewing metals mobilization by acidophilic microorganisms.

Some of the general oxidation reactions that acidophiles are able to catalyze are given below.

$$\text{Ferrous iron: } 2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

$$\text{General metal sulfide: } MS + 2O_2 \rightarrow M^{2+} + SO_4^{2-}$$

$$\text{Pyrite: } 4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$$

$$\text{Chalcopyrite: } CuFeS_2 + 4Fe^{3+} \rightarrow 5Fe^{2+} + Cu^{2+} + 2S^0$$

$$\text{Sulfur: } S^0 + 1\tfrac{1}{2}O_2 + H_2O \rightarrow H_2SO_4$$

These reactions not only solubilize the metals present in the minerals but also generate sulfuric acid. The acidophilic microorganisms, therefore, are able to stand not only low pH values but also very high metal concentrations. They possess heavy metal resistance or detoxification mechanisms. Neutrophilic bacteria have metal resistance mechanisms involving either an active efflux or a detoxification of metal ions by different transformations (**Figure 1**). In the case of copper, for example, these include intracellular complexation, decreased accumulation, extracellular complexation, or sequestration in the periplasm. Neutrophilic bacteria are able to grow in a range of copper concentration between 1 and $8\,\text{mmol}\,l^{-1}$ depending on the species. However, acidophiles such as *Acidithiobacillus ferrooxidans* or archaeons such as *Sulfolobus metallicus* can resist concentrations of copper up to 800 and $200\,\text{mmol}\,l^{-1}$, respectively. Most likely, they possess additional mechanisms to those present in neutrophiles, allowing them to have such dramatic metal resistance. It has been proposed that in microorganisms possessing large amounts of polyP, such as some chemolithoautotrophic acidophilic bacteria and archaea, these polymers may be actively involved in the elimination of toxic heavy metals such as Cu. This detoxification would take place through the enzymatic hydrolysis of polyP that would generate free phosphate that would bind the excess of cytoplasmic metal to form a metal–phosphate complex that is transported outside the cell through phosphate transporters (**Figure 1**). These properties make these microorganisms very appropriate for their use in biomining and also for the bioremedation or removal of heavy metals from polluted places (see below).

Microbial Extraction of Metals from Ores and Biomining

The microbial solubilization of metals is widely and successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium, and others. This process is done by using chemolithoautotrophic microorganisms. There is a great variety of microorganisms capable of growth in situations that simulate biomining commercial operations and many different species of microorganisms are living at acid mine drainage (AMD) sites. The most studied leaching bacteria are from the genus *Acidithiobacillus*. *A. ferrooxidans* (**Figure 2(b)**) and *Acidithiobacillus thiooxidans* are acidophilic mesophiles and together with the moderate thermophile *Acidithiobacillus caldus*, they belong to the Gram-negative γ-proteobacteria. **Figure 2(c)** shows *A. ferrooxidans* cells growing in the form of a biofilm on the surface of an elemental sulfur particle, most likely as a monolayer. When this biofilm is removed from the solid particle by using a detergent (**Figure 2(d)**), only those cells attached more strongly to the sulfur particle are remaining. They are seen as attacking the sulfur surface through a 'pitting' in which some cells are still tightly bound to the cavities and others have been released, leaving empty cavities. A similar attack has been observed on the surface of other minerals such as pyrite.

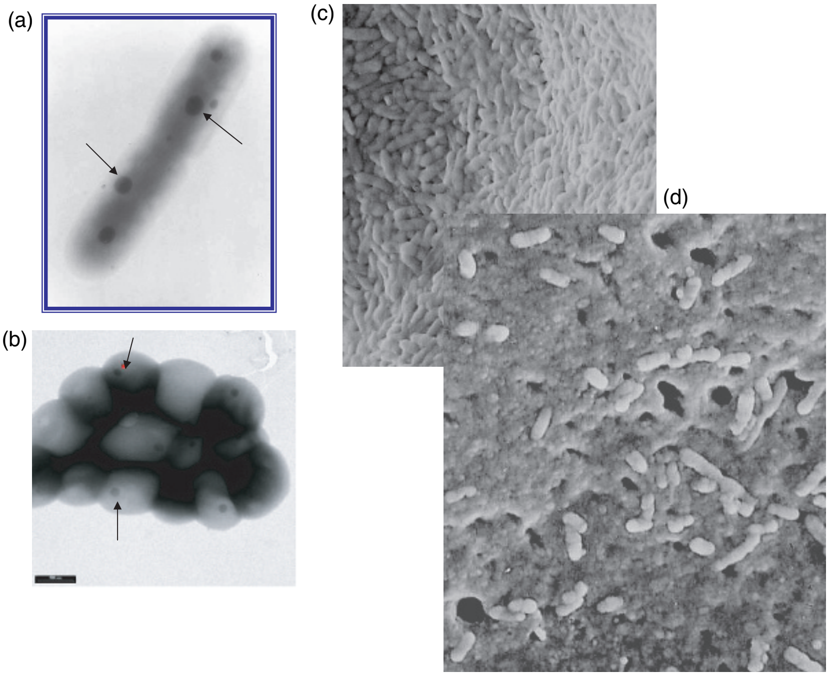

Figure 2 Some examples of acidophilic microorganisms that participate in metal extraction through biomining. (a) *Acidithiobacillus ferrooxidans* cells. (b) A group of *Sulfolobus metallicus* cells. (c) A biofilm, possibly a monolayer of *A. ferrooxidans* cells growing on the surface of an elemental sulfur prill. (d) Most of the biofilm of *A. ferrooxidans* seen in (c) was removed from the solid particle by using a detergent and vigorous shaking of the sample. The remaining cells seen are those more strongly adhered to the particle. Cells in (a) and (b) were observed by transmission electron microscopy (TEM) of unstained preparations and those in (c) and (d) by scanning electron microscopy. The arrows in each case point to electron dense polyphosphate granules that may help these microorganisms in their extremely high metal tolerance.

Members of the genus *Leptospirillum* are other important biomining bacteria that belong to a new bacterial division. Some Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferromicrobium*, and *Sulfobacillus* have also been described. Biomining using extremely thermophilic archaeons capable of oxidizing sulfur and iron (II) has been known for many years, and the archaeons are mainly from the genera *Sulfolobus* (**Figure 2(b)**), *Acidianus*, *Metallosphaera*, and *Sulfurisphaera*. Recently, some mesophilic iron (II)-oxidizing archaeons belonging to the Thermoplasmatales have been isolated and described – *Ferroplasma acidiphilium* and *Ferroplasma acidarmanus*. In fact, a consortium of different microorganisms participates in the oxidative reactions, resulting in the extraction of dissolved metal values from ores.

Industrial biomining operations are of several kinds depending on the ore type and its geographical location, the metal content, and the specific minerals present (metal oxides, metal sulfides of different kinds). One of the most used setups for the recovery of gold or copper is the irrigation type of processes. These involve the percolation of leaching solutions through the crushed ore that can be contained in a column, a heap or a dump. In **Figure 3**, we can see a scheme in which the crushed ore to bioleach is transported to an agglomeration tank or drum where it is acidified. This process is the key one since the bigger ore particles are surrounded by the very fine particles that stick to them thus preventing all the particles especially the fine material sediment to the bottom of the heap. In this way irrigation and aeration of the heap takes place from the top to the bottom, allowing a much more homogeneous growth of the microorganisms and therefore a better metal solubilization. The heap can be 6–10 m tall and 100 or more meters long and wide and is constructed over irrigation pads lined with high-density polyethylene to avoid losses of the pregnant copper-containing solution (**Figure 3**). This solution containing copper sulfate generated by the microbial solubilization of the insoluble copper sulfides present in the ore is subjected to solvent extraction to have a highly concentrated copper sulfate solution from which the metal is recovered in an electrowinning plant to generate electrolytic copper of high purity (**Figure 3**). Since most mining operations are located in areas where water is scarce, the spent leach liquors or raffinates are recirculated to the heap for further irrigation. This has to be controlled because the liquor is being enriched in salts that can rather select for those microorganisms able to stand the high salt and are not necessarily the most fit for the biooxidation reactions.

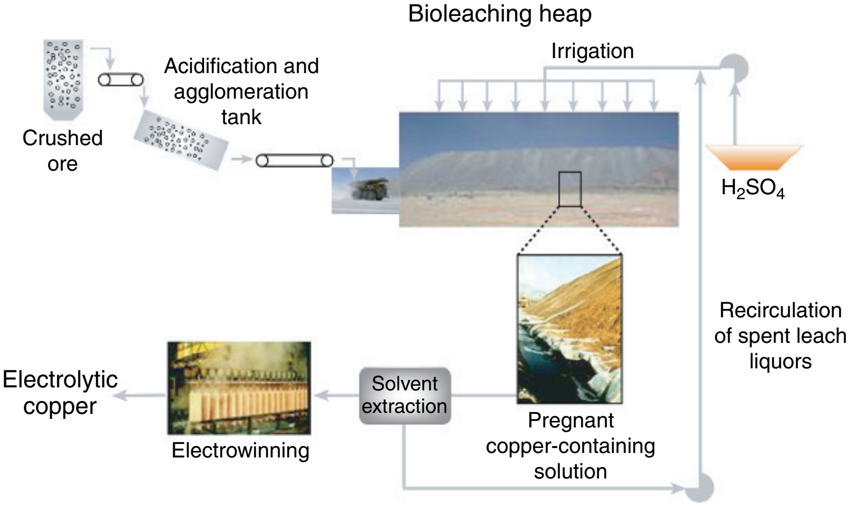
Bioleaching heap
Irrigation
Acidification and agglomeration tank
Crushed ore
H_2SO_4
Recirculation of spent leach liquors
Electrolytic copper
Solvent extraction
Pregnant copper-containing solution
Electrowinning

Figure 3 A scheme showing the construction of a heap bioleaching process to obtain copper in a large scale.

Bioleaching bacteria can also be used for gold recovery. Gold is usually found in nature associated with minerals containing arsenic and pyrites (arsenopyrites). During gold bioleaching, the iron- and sulfur-oxidizing microorganisms attack and solubilize the arsenopyrite releasing the trapped gold particles. Following this release, the gold is complexed with cyanide according to standard gold-mining procedures. Instead of using big leaching heaps or dumps as in the case of bioleaching of copper ores, gold bioleaching is usually done by using highly aerated stirred tank bioreactors connected in series. Since these reactors are expensive to build, they are used with high-grade ores or with mineral concentrates. The advantage of tank reactors over heaps and dumps, which are 'open bioreactors', is that in the tanks conditions can be controlled, thus having a much faster and efficient metal extraction process.

Currently, there are operations using both mesophilic and thermophilic microorganisms. Biomining has distinctive advantages over the traditional mining procedures. For example, it does not require the high amounts of energy used during roasting and smelting and does not generate harmful gases such as sulfur dioxide. Nevertheless, AMD can be generated, which if not properly controlled, pollutes the environment with acid and metals. Biomining is also of great advantage since not only discarded low-grade ores from standard mining procedures can be leached in an economically feasible way but also some high-grade ores. In countries like Chile, which is actually the first world copper producer, many mining operations process from 10 000 to 40 000 tons of ore per day and produce between 10 000 and 200 000 tons of copper per year by using heap or dump bioleaching of minerals such as oxides, chalcocite, covellite, chalcopyrite, and others. Similar situations take place in United States, Australia, and other countries. The most successful ones have been those processing copper oxides and secondary copper sulfides. However, chalcopyrite is the most abundant copper sulfide in the world. Since it is the most difficult to be solubilized by microorganisms, there is actually great interest in developing processes mainly using thermophilic biomining microorganisms.

Acidiophilic Microorganism–Mineral Interaction

The microorganisms used in biomining belong to those known as extremophiles since they live in extremely acidic conditions (pH 1–3.0) and in the presence of very high toxic heavy metals concentrations. Considerable effort has been spent in the past few years to understand the biochemistry of iron and sulfur compounds oxidation, bacteria–mineral interactions, and the several adaptive responses that allow the microorganisms to survive in a bioleaching environment. All of these are considered key phenomena for understanding the process of biomining.

How do the bacterial cells recognize the site of attachment in the ores from which they will extract the metals? Do they have a specific way to detect or sense where the oxidizable substrate is present in the rocks? Ample evidence has shown that attachment of the bacterial cells to metal sulfides does not take place randomly. For example, *A. ferrooxidans* cells preferentially adhere to sites with visible surface scratches and it is seen to form pitting at the surface of minerals as already seen in **Figure 2**.

Bacteria such as *A. ferrooxidans* and *Leptospirillum ferrooxidans* have been shown to possess a chemosensory system that allows them to have chemotaxis, that is, the capacity to detect gradients of oxidizable substrates being extracted from the ores such as Fe (II)/Fe (III) ions, thiosulfate, and others (**Figure 4**). The response is the positive

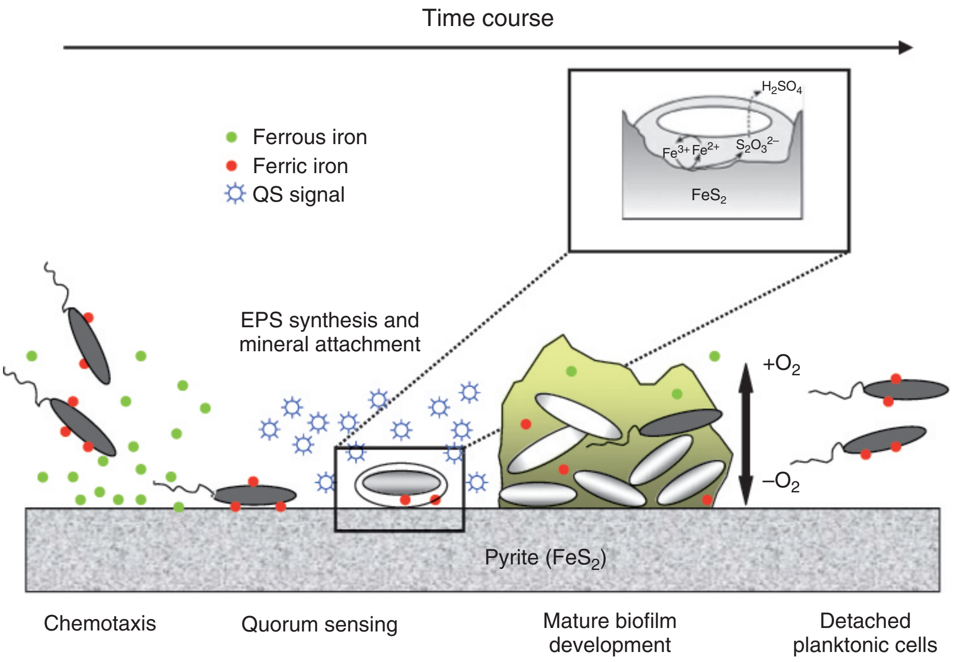

Figure 4 A diagrammatic representation showing the main steps of the bacteria–mineral interaction. Details of the attack of *Acidithiobacillus ferrooxidans* on the mineral pyrite are shown in the amplified inset. The cell is embedded in an extracellular polymeric substances (EPS) biofilm in which the indirect mechanism generates ferrous iron and thiosulfate, which is finally oxidized to sulfuric acid. Inset Reproduced from Rohwerder T, Gehrke T, Kinzler K, and Sand W (2003). Bioleaching review part A: Progress in bioleaching: Fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied Microbiology and Biotechnology* 63: 239–248.

chemotactic attraction to the sites that will constitute specific favorable mineral attachment sites.

It is known that most leaching bacteria grow attached to the surface of the solid substrates such as elemental sulfur and metal sulfides. This attachment is predominantly mediated by extracellular polymeric substances (EPS) surrounding the cells and whose composition is adjusted according to the growth substrate (**Figure 4**). Thus, planktonic or free-swimming cells grown with soluble substrates such as iron (II) sulfate produce almost no EPS.

Biofilms are organized layers of bacteria associated to a solid surface by means of the matrix of EPS. The environment within this community favors intercellular interactions between bacteria. Thus, in the presence of oxygen or aerobiosis, *A. ferrooxidans* can respire this element and oxidize Fe (II). Some of these microorganisms are also able to perform Fe (III) respiration under anaerobiosis, regenerating Fe (II) that can be used by those microbial cells closer to the oxygen within the biofilm structure. Other types of bacteria forming part of the microbial biofilm consortium will generate compounds useful to other members of the community and they can themselves benefit from the metabolic byproducts of other microbes present in the biofilm.

Several microorganisms are known to monitor their own population density through processes collectively described as quorum sensing (QS), and in several cases there is strong evidence indicating that biofilm formation is affected by QS. In most cases, specific genes within the bacterium are switched on at a defined population density (defined bacterial quorum) and the result obtained is the activation of functions under the control of a quorum sensor. In almost all cases, the capacity to detect a bacterium quorum depends on the release of a signal molecule from the microorganism that accumulates in proportion to the cell number (**Figure 4**). Thus, QS represents a multicellular action in bacteria, where each cell communicates with each other to coordinate their behavior. Very recently, it has been demonstrated that *A. ferrooxidans* not only contains quorum sensor signal molecules but may induce the expression of genes related to QS and EPS production when grown attached to a solid substrate. Thus, modulation of the attachment of the microorganisms to ores through interferences of their QS responses can be envisaged as a new way to control metal extraction by these microorganisms.

A. ferrooxidans is also able to develop biofilm structures when growing in solid substrates such as elemental sulfur or metal sulfides and presents morphological modifications during the cellular adhesion process. For example, new proteins related to sulfur metabolism appear in the surface of *A. ferrooxidans* when grown in sulfur. This is clearly seen in **Figure 5**, in which a primary antibody specific against a protein related to sulfur metabolism is bound to the surface of *A. ferrooxidans* cells grown in sulfur

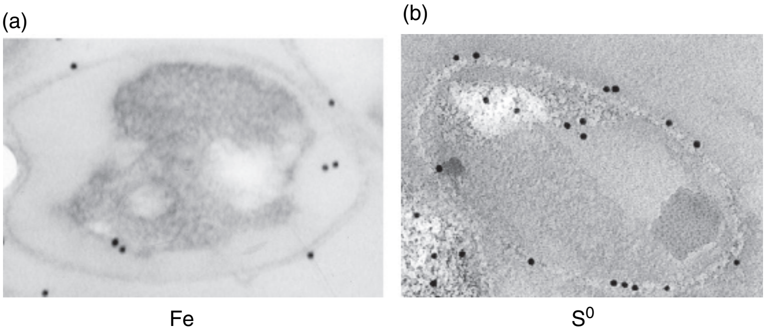

Figure 5 Example of changes on the *Acidithiobacillus ferrooxidans* surface depending on growth conditions. It is known that *A. ferrooxidans* produces a protein in high amounts when the bacterium grows on elemental sulfur and not in ferrous iron (Fe). To see the location of that protein, an immunological system was used in which a gold particle (black dots) indicates the presence of the protein in the cells grown in ferrous iron (Fe) (a) or elemental sulfur (b).

but is almost absent in ferrous iron-grown cells. The primary antibody bound to the protein was recognized by using a secondary antibody labeled with gold particles and specific to recognize the primary antibody bound to the sulfur-induced protein. The presence of the protein changing its expression is seen as the black dots of gold by transmission electron microscopy (TEM).

Mechanisms Involved in Metal Solubilization by Acidophiles

Traditionally, it has been proposed that microorganisms oxidize metal sulfides by either a direct or an indirect mechanism. In the first one, the bacteria have to attach to the mineral and the electrons would be directly extracted by an enzymatic reaction of the microorganism. However, there is no evidence how the bacteria break the metal–sulfide bond of a given mineral. In the indirect mechanism, the role of bacteria would be to oxidize ferrous ions present in the solution to ferric ions. Ferric ions are strong oxidants therefore will oxidize chemically the sulfide metals, being an indirect mode of attack. Actually, many researchers consider the existence of a contact mechanism, since the bacteria attaches to the surface of the mineral carrying Fe (III) bound to its exopolysaccharides, and when the microorganism forms a biofilm being embedded in its EPS, this metal would chemically attack the metal sulfide generating, in the case of pyrite, ferrous iron that is reoxidized to Fe (III) and thiosulfate that can be further oxidized to sulfuric acid (**Figure 4**). The mechanism still is indirect (known also as indirect contact mechanism). This close contact of the bacterium with the mineral makes more efficient and specific the sulfide oxidation.

The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the reoxidation of the generated ferrous iron to obtain additional ferric iron (**Figure 4**).

As already mentioned, *A. ferrooxidans* is a chemolithoauthotrophic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds and it has been considered as a model biomining microorganism. The reactions involved in ferrous iron oxidation by *A. ferrooxidans* have been studied in detail, however, the electron pathway from ferrous iron to oxygen has not been entirely established. The terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin and cytochrome c552. Recently, a high molecular weight c-type cytochrome, Cyc2, has been suggested to be the prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (**Figure 6**). This pathway would be $\text{Cyc2} \rightarrow \text{rusticyanin} \rightarrow \text{Cyc1(c552)} \rightarrow \text{aa3}$ cytochrome oxidase. In addition, there is an apparent redundancy of electron transfer pathways via bc(1) complexes and terminal oxidases in *A. ferrooxidans*.

As already mentioned, thiosulfate has been postulated as a key compound in the oxidation of the sulfur moiety of pyrite (**Figure 4**). Iron (III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental sulfur being a side product. This explains why only Fe(II) ion-oxidizing bacteria are capable of oxidizing these metal sulfides. All reactions comprising this oxidation have been shown to occur chemically. However, sulfur compound-oxidizing enzymes such as the tetrathionate hydrolase of *A. ferrooxidans*, *A. thiooxidans*, or *T. acidophilus* may also be involved in the process.

The oxidation of some metal sulfides such as chalcopyrite generates elemental sulfur as a side product instead of thiosulfate (**Figure 6**). The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (SDO) and a sulfite oxidoreductase (SOR) (**Figure 6**).

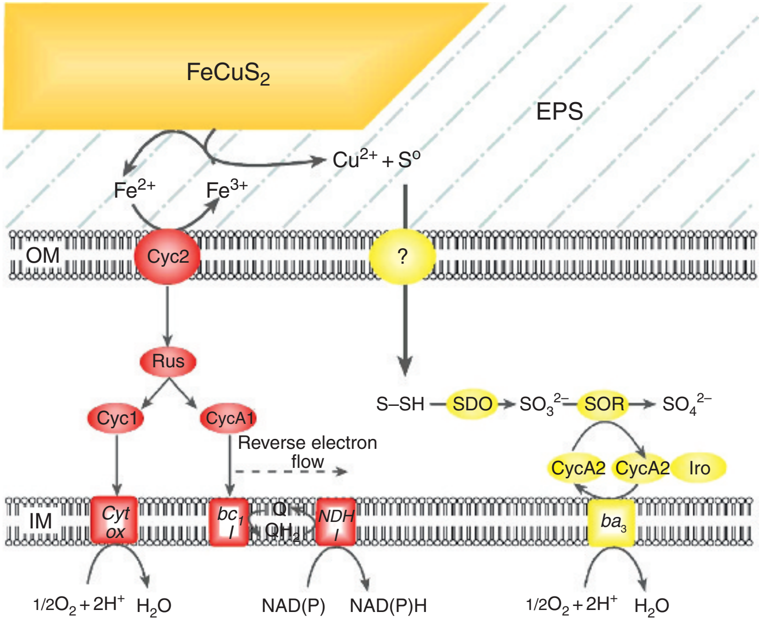

Figure 6 Simplified model showing the main protein components (in red) present in the periphery of the cell and involved in the transfer of electrons from ferrous iron to oxygen and those oxidizing elemental sulfur (in yellow) and transferring the electrons to oxygen. This model represents the indirect mechanism of mineral attack by the ferric iron bound to the EPS of the *Acidithiobacillus ferrooxidans* cells. As an example, chalcopyrite is shown from which ferrous iron, copper (II), and elemental sulfur are generated upon its oxidation. SDO, sulfur dioxygenase; SOR, sulfite oxidoreductase. Adapted from Rawlings DE (2005) Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microbial Cell Factories* 4: 13.

It is important to remark here that the ultimate oxidizing agent for iron (II) and reduced inorganic sulfur compounds is oxygen, since often the transport of dissolved oxygen is the rate-limiting step in commercial bioleaching operations.

Biomining in the Postgenomic Era

Systems microbiology, which is part of systems biology, is a new way to approach research in biological systems. By this approach, it may be possible to explore the new properties of microorganisms that arise from the interplay of genes, proteins, other macromolecules, small molecules, and the environment. This is particularly possible today due to the large numbers of genomic sequences that are becoming increasingly available. However, additional genomic sequences of the different biomining microorganisms will be required to define the molecular adaptations to their environment and the interactions between the members of the community.

The use of genomics, metagenomics, proteomics and metaproteomics (**Figure** 7), together with metabolomics to study the global regulatory responses that the biomining community uses to adapt to their changing environment is just beginning to emerge. These powerful OMICS approaches will have a key role in understanding the molecular mechanisms by which the microorganisms attack and solubilize ores. Furthermore, they offer the possibility of discovering exciting new findings that will allow analyzing the community as a microbial system, determining the extent to which each of the individual participants contributes to the process, and how they evolve in time to keep the conglomerate healthy. This, taken together with the physicochemical, geological, and mineralogical aspects of the process, will allow improving the efficiency of this important biotechnology.

Biomining Community Diversity Analysis

Of extreme importance is not only to know the microorganisms present in a bioleaching operation, but to be able to monitor their behavior during the process, to determine the predominant species and the way they evolve in time with the changing environment as the metals are solubilized. In recent years, several molecular methods have been developed for other microorganisms and these have been successfully applied to many biomining operations. The most common techniques employed to explore the bacterial diversity use 16S rRNA and rDNA profiles and are culture-independent methods. Among these methods are included fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), real time PCR, DNA microarrays, and others (**Figure** 7).

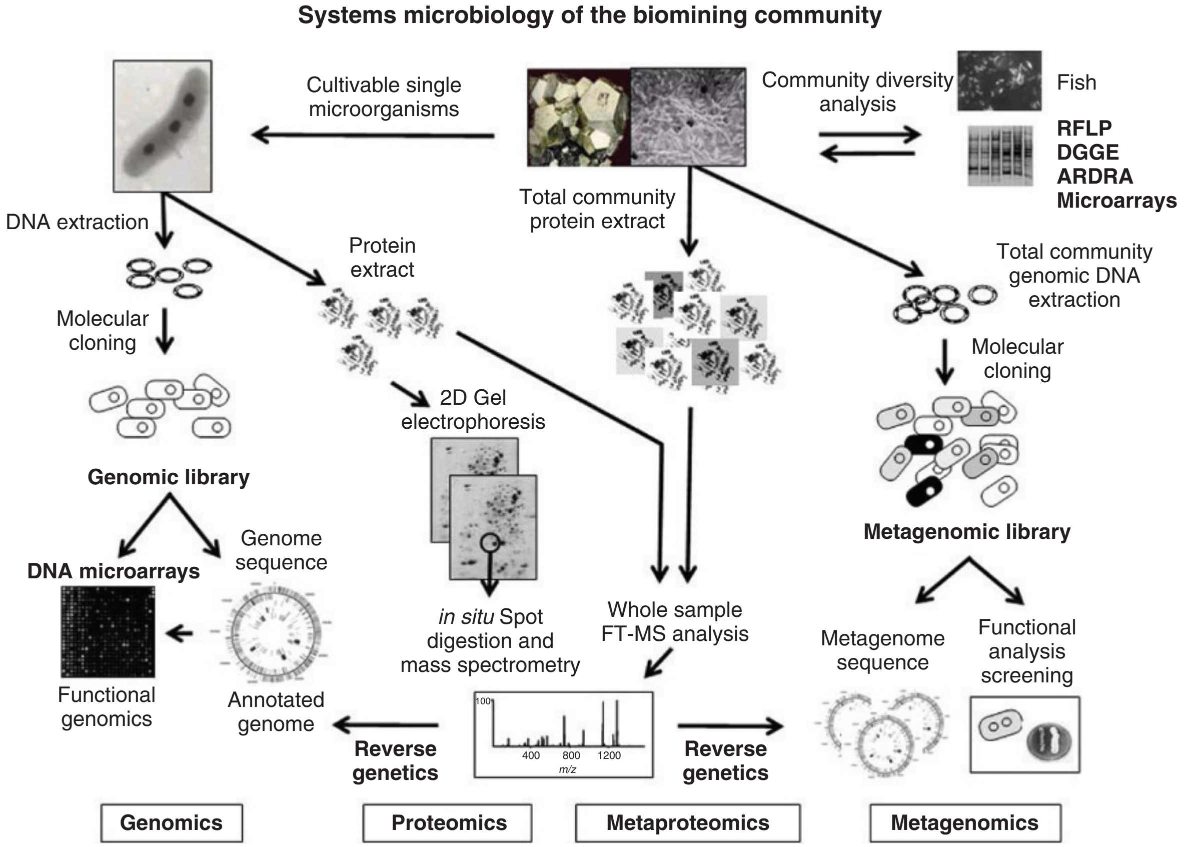
Systems microbiology of the biomining community
Cultivable single microorganisms
Community diversity analysis
Fish
RFLP
DGGE
ARDRA
Microarrays
DNA extraction
Protein extract
Total community protein extract
Total community genomic DNA extraction
Molecular cloning
2D Gel electrophoresis
Molecular cloning
Genomic library
Metagenomic library
DNA microarrays
Genome sequence
in situ Spot digestion and mass spectrometry
Whole sample FT-MS analysis
Metagenome sequence
Functional analysis screening
Functional genomics
Annotated genome
Reverse genetics
100
400
800
1200
m/z
Reverse genetics
Genomics
Proteomics
Metaproteomics
Metagenomics

Figure 7 Systems microbiology and the use of OMICS approaches to study microbial communities as applied to biomining microbes. Modified from Valenzuela L, Chi A, Beard S, *et al*. (2006). Genomics, metagenomics and proteomics in biomining microorganisms. *Biotechnology Advances* 24: 197–211.

Genomics

A. ferrooxidans was the first biomining microorganism whose genome was sequenced by TIGR. This information has been very useful to do genome-wide searches for candidate genes for important metabolic pathways and several important physiological functions, which can now be addressed. Furthermore, predictions for the functions of many new genes can be done. The main focus of research has been the energy metabolism that is directly responsible for bioleaching. Genes involved in phosphate, sulfur, and iron metabolism, QS, those potentially involved in several other functions such as metal resistance and amino acid biosynthesis pathways, and those involved in the formation of EPS precursors have been studied. Having the genomic sequence of a given biomining microorganism is very important, since it is then possible to formulate hypothesis about the regulation of the expression of most of these genes under different environmental conditions. Metabolic reconstruction and modeling provides an important preliminary step in understanding the unusual physiology of this extremophile especially given the severe difficulties involved in its genetic manipulation and biochemical analysis. However, all these bioinformatic predictions will have to be demonstrated experimentally by using functional genomics, proteomics, and other approaches. The existence of paralog genes that show sequence similarities but may have different functions in the same microorganism becomes obvious only when the genome sequence is available.

So far, there are few genomic sequences of acidophilic microorganisms usually found in biomining or AMD places. These are for the bacteria *A. ferrooxidans*, *Leptospirillum* group II, and *Acidiphilium cryptum.* It has also been mentioned that although not yet publicly available, the genome sequences of *A. thiooxidans* and *A. caldus* have been determined. Amongst the archaea, *Metallosphaera sedula* and *F. acidarmanus* genome sequences are available. *Sulfolobus acidocaldarius* and *Sulfolobus tokodaii* are also known, although they have not been reported as having a role in bioleaching. It is therefore not difficult to predict in the very near future the generation of new DNA microarrays to monitor not only all the microorganisms present in samples from industrial operations (**Figure 7**) but also specific genes such as those indicating the nutritional or stress state of the microorganisms or those involved in iron or sulfur compound oxidation whose products will be predominant during different stages of active bioleaching.

Functional genomics

One of the most used techniques to study differential genome expression at the level of mRNA synthesis, transcriptomics, or the functional analysis of new and characterized genes is the use of DNA microarrays. However, a prerequisite to apply microarrays is to know the genomic sequence of the organism to be analyzed.

The use of microarrays based on the entire genome of *A. ferrooxidans* and other microorganisms will enable a nearly complete view of gene expression of the members of the microbial community under several biomining conditions, helping to monitor their physiological state and adjustment made during the bioleaching process. A first preliminary pilot DNA macroarray formed with 70 different genes has been used to study the relative variations in mRNA abundance of some genes related with sulfur metabolism in *A. ferrooxidans* grown in different oxidizable substrates. A genome-wide microarray transcript profiling analysis (approximately 3000 genes of the *A. ferrooxidans* ATCC 23270 strain) has also been performed. The genes preferentially transcribed in ferrous iron growth conditions or in sulfur conditions were studied. The results obtained supported and extended models of iron and sulfur oxidation (**Figure 6**) and supported the possible presence of alternate electron pathways and that the oxidation of these two kinds of oxidizable substrates may be coordinately regulated. By using the same approach, the expression of the genes involved in carbon metabolism of *A. ferrooxidans* has been studied in response to different oxidizable substrates.

As already mentioned, some mining companies are currently interested in doing transcriptomic analysis of their newly isolated microorganisms with improved capacities to leach copper since they already have obtained their genomic sequences.

A very interesting alternative approach can be used to analyze gene function in environmental isolates without knowing the sequence of the microorganism of interest. A random genomic library from the isolated microorganism can be printed on a microarray. Gene expression by using total RNA extracted from the microorganism grown under different conditions can be determined. With this approach, it is possible to select and sequence only those clones bearing the genes that showed an altered expression pattern. Shotgun DNA microarrays are very powerful tools to study gene expression with environmental microorganisms whose genome sequence is still unknown.

In the near future, the use of microarrays based on the entire genomes of biomining microorganisms will allow having a nearly complete view of gene expression of the members of the microbial community under several biomining conditions, helping to monitor their physiological state and adjustment made during the bioleaching process.

Metagenomics

Metagenomics is the culture-independent genomic analysis of microbial communities. In conventional shotgun sequencing of microbial isolates, all shotgun fragments are derived from clones of the same genome. To analyze the genomes of an environmental microbial community (**Figure 7**), the ideal situation is to have a low diversity environment. Such systems were found when analyzing the microbial communities inhabiting a site of extreme AMD production, in which few types of organisms were present. Still, variation within each species might complicate assembly of the DNA fragments. Nevertheless, random shotgun sequencing of DNA from this natural acidophilic biofilm was used. It was possible to reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partially recover three other genomes. The extremely acidic conditions of the biofilm (pH about 0.5) and relatively restricted energy source combine to select for the small number of species found.

The analysis of the gene complement for each organism revealed the metabolic pathways for carbon and nitrogen fixation and energy generation. For example, genes for biosynthesis of isoprenoid-based lipids and for a variety of proton efflux systems have been identified, providing insights into survival strategies in the extreme acidic environment. Clearly, the metagenomic approach for the study of microbial communities is a real advancement to fully understand how complex microbial communities function and how their component members interact within their niches. A full understanding of the biomining community also will require the use of all these current molecular approaches.

Proteomics

Proteomics provide direct information of the dynamic protein expression in tissue or whole cells, giving us a global analysis. Together with the significant accomplishments of genomics and bioinformatics, systematic analysis of all expressed cellular components has become a reality in the post genomic era, and attempts to grasp a comprehensive picture of biology have become possible.

One important aspect of proteomics is to characterize proteins differentially expressed by dissimilar cell types or cells imposed to different environmental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with mass spectrometry (MS) is currently the most widely used technology for comparative bacterial proteomics analysis (**Figure 7**). The high reproducibility of 2D-PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms.

2D-PAGE separates a complex mixture of proteins such as those present in a bacterial cell extract based on the isoelectric point of the proteins in the first dimension (isoelectrofocusing gel). These proteins or groups of proteins are further resolved in a second dimension (SDS-PAGE), in which the proteins are all negatively charged and are separated based on their molecular masses (**Figure 7**). Actual 2D-PAGE procedures can resolve around 1000 protein spots in a single run. One of the limitations of 2D-PAGE is that only the most abundant proteins in the cell can be detected. Therefore, to increase the resolution of the method, it is also possible to analyze a subproteomic cell fraction instead of the total cell proteins as shown for the periplasmic proteins from *A. ferrooxidans*.

The high reproducibility of 2D-PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms. This is a typical 'reverse genetics' approach in which (after isolating from a 2D-PAGE gel) an individual protein is differentially expressed in a condition of interest, and the amino acid sequence of a peptide from the protein is obtained to identify its possible homologue in databases. With this information, its coding gene and genomic context can be searched using the genome DNA sequence (**Figure 7**). Depending on these results, a suggested function could be hypothesized. It will be of great importance to demonstrate the expression of putative genes related to EPS synthesis in *A. ferrooxidans* cells grown on different metal sulfides and to find out if these genes are involved in cell attachment and biofilm formation. In this regard, *A. ferrooxidans* is known to form biofilms on solid substrates (**Figure 2**). In an AMD biofilm analyzed by the proteomic approach, it was not known which microorganisms are responsible for the production of the polymer embedding the community. However, the presence of numerous glycosyltransferases and polysaccharide export proteins in the predominant bacterial species also suggests a role in biofilm formation.

Several studies have used 2D-PAGE to study changes in protein expression of *A. ferrooxidans* under different growth conditions. Proteins induced under heat shock, pH stress, phosphate limitation, or the presence of copper have been reported. A set of proteins that changed their levels of synthesis during growth of *A. ferrooxidans* in metal sulfides, thiosulfate, elemental sulfur, and ferrous iron has been characterized by using 2D-PAGE.

During growth of *A. ferrooxidans* in metal sulfides containing iron, such as pyrite and chalcopyrite, proteins upregulated both in ferrous iron and in sulfur compounds were synthesized, indicating that the two energy-generating pathways are simultaneously induced depending on the kind and concentration of the available oxidizable substrates.

In the past decade, an increasing number of sequenced genomes provided good options for high-throughput functional analysis of proteomes. Proteomic studies are well advanced for diverse bacteria, such as the model bacterium *Escherichia coli* and others. Nevertheless, there is still a lack of data for identification of proteins from organisms with unannotated or unsequenced genomes, which makes large-scale microbacterial proteomics analysis a challenge. With the development of highly sensitive and accurate computational gene-finding methods, new microbial genomes could be explored and scientific knowledge of them could be maximized.

Traditional 2D gel electrophoresis coupled with MS is time consuming as a result of the nature of spot-by-spot analysis and it is biased against low abundance proteins, integral membrane proteins, and proteins with extreme pI or molecular weight (MW). Alternatively, solution-based approaches offer unbiased measurement of relative protein expression regardless of their abundance, subcellular localization, or physicochemical parameters (**Figure 7**). This methodology, however, results in extremely complex samples. For instance, of the 4191 predicted genes in the complete genome of *E. coli*, 2800 of them are believed to be expressed at any one time. Additional complexity is introduced upon enzymatic digestion, which generates multiple peptide species for each protein. To obtain comprehensive protein expression information from the samples, a chromatographic separation step prior to MS protein analysis is often necessary. High performance liquid chromatography (HPLC) coupled with online electron spray ionization MS (ESI-MS/MS) has been proved to be a valid approach for analyzing protein expression in complex samples. Alternatively, Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) is well suited for the differential analysis of protein expression due to the high mass accuracy and high resolution as well as its inherent wide dynamic range. Peptide charge state can be readily derived with the accurate isotopic peak distribution information provided by FT MS experiment, and co-eluting species with the same nominal m/z ratio can be resolved. The differentially expressed m/z values identified can be assigned to the peptide sequences and, subsequently, differentially expressed proteins can be identified (**Figure 7**).

In the periplasm of *A. ferrooxidans*, 216 proteins were identified, several of them changing their levels of synthesis when the bacterium was grown in thiosulfate, elemental sulfur, or ferrous iron media. Thirty four percent of them corresponded to unknown proteins. Forty one proteins were exclusively present in sulfur-grown and 14 in thiosulfate-grown cells. The putative genes coding for all the proteins were localized in the available genomic sequence of *A. ferrooxidans* ATCC 23270. The genomic context around several of these genes suggests their involvement in sulfur metabolism and possibly in sulfur oxidation and formation of Fe–S clusters.

Metaproteomics

Recently, the term 'metaproteomics' was proposed for the large-scale characterization of the entire protein complement of environmental microbiota at a given point in time. High-throughput MS has been used in a metaproteomic approach to study the community proteomics in a natural AMD microbial biofilm. Two thousand and thirty-three proteins from the five most abundant species in the biofilm were detected, including 48% of the predicted proteins from the dominant biofilm organism *Leptospirillum* group II. It was also possible to determine that one abundant novel protein was a cytochrome central to iron oxidation and AMD formation in the natural biofilm. This novel approach together with functional metagenomics can offer an integrated study of a microbial community to establish the role each of the participant plays and how they change under different conditions.

The goal of functional proteomics is to correlate the identification and analysis of distinct proteins with the function of genes or other proteins. With the discovery of a variety of modular protein domains that have specific binding partners, it has become clear that most proteins occur in protein complexes and that the understanding of a function of a protein within the cell requires the identification of its interacting partners. In the case of a biomining bacterium such as *A. ferrooxidans*, the identification of protein complexes involved in oxidative reactions is of high priority. What complexes are formed by cytochrome-like proteins such as rusticyanin (Rus in **Figure 6**) with other proteins in the periplasm? Do some periplasmic proteins form a complex involved in sulfur compound oxidation in the periplasm? What other complexes of oxidative reactions are present in this microorganism? Proteomics may answer these and many other questions that will help to understand better the biomining process.

The OMICS procedures briefly analyzed and summarized in **Figure 7** should be used in close conjunction with the known physiological functions of the microorganisms being studied. It should be possible to better control the activity of the bacteria and archaea by giving them the appropriate nutritional and physicochemical conditions, and by interfering with some of these microbiological functions in order to enhance their action (for metal extraction) in the case of biomining or to inhibit their capacities to control AMD. As already mentioned (**Figure 4**), some of these key physiological behaviors are chemotaxis, QS, and biofilm formation. Bacteria such as *A. thiooxidans* and *L. ferrooxidans* clearly possess chemotactic systems and are attracted by a concentration gradient of thiosulfate or ferrous iron such as the one generated on the surface of pyrite (**Figure 4**). This sensing ability is very important for the specific bacterial adherence at the places where the substrates to be solubilized through oxidation are present.

The development of efficient transformation or conjugation systems to introduce DNA to generate mutations affecting a gene of interest by using gene knockout systems is almost entirely lacking for biomining microorganisms. These tools will be essential not only to perform functional genomics and have experimental demonstrations for the suggested gene functions based on bioinformatics analysis of the postgenomic data, but also to eventually improve some physiological bacterial capabilities. At the same time, the new OMICS methods are greatly helping to monitor more precisely the biomining consortia, and it is expected that providing the right physiological conditions to the community together with proper chemical or physical manipulations of the bacterial environment will further improve bioleaching rates in biomining operations.

Environmental Effects of Metals Solubilization and Bioremediation

The acidophilic microorganisms mentioned, mobilize metals and generate AMD, causing serious environmental problems. AMD should be remediated or abated. Often there is a sealing of the contaminated sites or the location of barriers to contain the acidic fluids. Many approaches use prevention techniques to avoid further spillage of acidic effluents in the contaminated area. It can be controlled by chemical treatments such as the use of calcium oxide that neutralizes the acid pH. It is also possible to inhibit the acidophilic microorganisms responsible of the acid generation. This can be done by using certain organic acids, sodium benzoate, sodium lauryl sulfate, or quaternary ammonium compounds that will affect the growth of bacteria such as *A. ferrooxidans*.

Bioremediation or removal of the toxic metals from these contaminated soils can be achieved by a very interesting combination of two opposite biological activities: that of sulfur-oxidizing bacteria with the one of sulfate-reducing microorganisms. In a first step, the sulfur-oxidizing bacteria generate sulfuric acid which bioleaches or solubilizes the metals in the solid phase of the soil. The leachate metals are then precipitated in a second step by using a bioreactor in which the hydrogen sulfide generated by the sulfate-reducing bacteria under neutral and anaerobic conditions forms insoluble metal sulfides, which are eliminated (**Figure 8**). Metal contaminants such as Cu, Cd, Ni, and others can be efficiently leached from contaminated soils. The effluents obtained from such a process are clean enough of the metals that they can be reused in the environment.

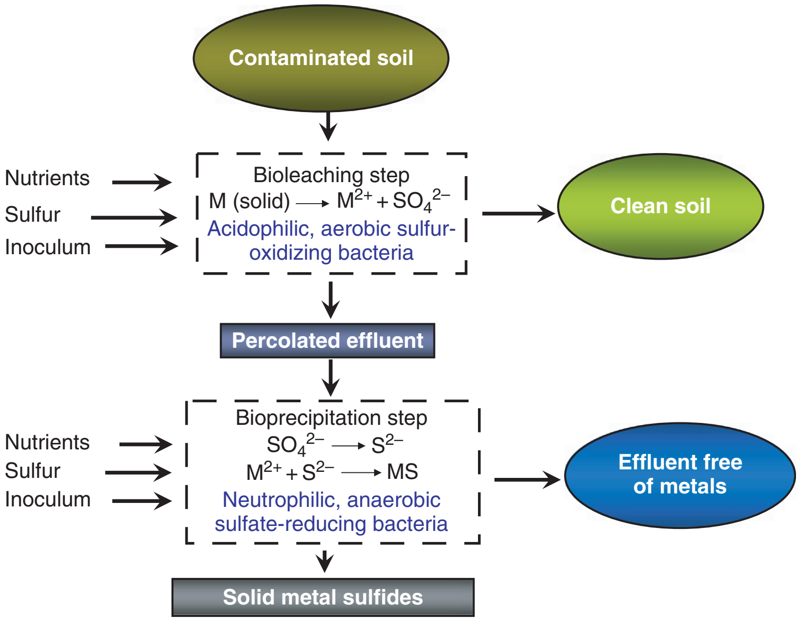
Contaminated soil
Nutrients
Sulfur
Inoculum
Bioleaching step
M (solid) ⟶ $M^{2+} + SO_4^{2-}$
Acidophilic, aerobic sulfur-oxidizing bacteria
Clean soil
Percolated effluent
Nutrients
Sulfur
Inoculum
Bioprecipitation step
$SO_4^{2-} \longrightarrow S^{2-}$
$M^{2+} + S^{2-} \longrightarrow MS$
Neutrophilic, anaerobic sulfate-reducing bacteria
Effluent free of metals
Solid metal sulfides

Figure 8 A schematic diagram illustrating a process for the bioremediation of soils polluted with metals.

Bioleaching microorganisms such as *A. ferrooxidans* can also have other uses to help avoiding metal contaminations in modern societies. For example, this bacterium has been successfully used to recover metals such as cadmium from spent batteries. By using bioreactors, *A. ferrooxidans* is grown attached on elemental sulfur. The bacteria generate sulfuric acid through the oxidation of sulfur that is then used for the indirect dissolution of spent nickel–cadmium batteries recovering after 93 days 90–100% of cadmium, nickel, and iron. Bioleaching of spent lithium ion secondary batteries, containing lithium and cobalt, has also been explored. These approaches are not only economically valuable but may be an effective method which could be considered the first step to recycle spent and discarded batteries preventing one of the many problems of environmental pollution.

Conclusions

Microorganisms (Bacteria and Archaea) require several metals that are essential for their life. However, when the metal concentrations reach toxic levels, they also possess metal resistance mechanisms that involve active efflux or a detoxification of metal ions by different transformations. They can transform most toxic metals to less soluble or less volatile forms by intracellular complexation, decreased accumulation, extracellular complexation, or sequestration in the periplasm. Some of these activities result in the solubilization or extraction of metals, which are successfully used in environmental biotechnological applications.

The aerobic mineral oxidation or the anaerobic respiration of different microorganisms can result in the solubilization or extraction of metals. All these bacteria–metal interactions are part of the normal biogeochemical processes in nature.

The microbial solubilization of metals in acid environments is successfully used in industrial processes called bioleaching of ores or biomining, to extract metals such as copper, gold, uranium and others. On the contrary, the acidophilic microorganisms mobilize metals and generate AMD, causing serious environmental problems. However, bioremediation or removal of the toxic metals from contaminated soils can be achieved by using the specific properties of microorganisms interacting with metals.

Current approaches to study microorganisms consider the microorganism or the community as a whole, integrating fundamental biological knowledge with genomics, proteomics, metabolomics, and other data to obtain a global picture of how a microbial cell or a community functions. This new knowledge will help not only in understanding microbiological phenomena but it will be also useful to improve applied microbial biotechnologies.

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Relevant Website

http://cmr.jcvi.org – Comprehensive Microbial Resource

Mycoplasma and *Spiroplasma*

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Defining Statement
Introduction
The Systematics of the Mollicutes
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Genetics and Molecular Biology of the Mollicutes
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Glossary

adhesin Bacterial protein that is involved in the adhesion to the eukaryotic host cell.

firmicutes A monophyletic group of Gram-positive bacteria whose genomes possess low GC content. The mollicutes are a subgroup of the firmicutes.

phosphotransferase system (PTS) A multicomponent system for the transport of carbohydrates into the bacterial cell. The PTS is composed of two general energy-coupling proteins and a set of sugar-specific permeases. The incoming sugars are phosphorylated at the expense of phosphoenolpyruvate concomitant with their transport.

promoter A DNA sequence upstream of a gene that is recognized and bound by the RNA polymerase and used to initiate transcription.

synthetic biology A new field of biology that is devoted to the generation of novel creatures and to the experimental verification of our concepts of life.

terminal organelle (tip structure) A complex cell structure characteristic of the mollicutes. The terminal organelle is required for cell division, movement, and adhesion to the host cell.

Abbreviations

AY-WB	aster yellows *Phytoplasma* strain witches broom
LC	large colony
MMR	multiple mutation reaction
OY-M	onion yellows strain
PCR	polymerase chain reaction
PTS	phosphotransferase system
TCA cycle	tricarboxylic acid cycle

Defining Statement

Mycoplasma and *Spiroplasma* species are bacteria that lack a cell wall (the mollicutes). These organisms evolved in close association with their eukaryotic hosts, resulting in an extreme genome reduction. In this article, the biology of the mollicutes is discussed with special emphasis on their pathogenicity, cell biology, and molecular biology.

Introduction

Mycoplasmas and spiroplasmas are two important genera of the bacterial group called mollicutes. The name mollicutes – *soft skin* – reflects the major collective characteristic of these bacteria – the lack of a cell wall – which at the same time distinguishes them from all other bacteria with the exception of the chlamydiae. The lack of a cell wall is caused by the absence of genes encoding enzymes for peptidoglycan biosynthesis. The lack of a cell wall is closely linked to another characteristic feature of the mollicutes – their cells are usually pleomorphic. Again, there is no rule without exception: the cells of the genus *Spiroplasma* have a helical shape (see following text).

Another important feature of the mollicutes is their close association with eukaryotic host organisms. In nature, mollicutes are never found as free-living organisms. Hosts are either animals including humans (*Mycoplasma*, *Ureaplasma*) or plants and insects (*Spiroplasma*, *Phytoplasma*) (**Table 1**). *Mycoplasma* species usually cause mild diseases such as atypical pneumonia (*Mycoplasma pneumoniae*) or nongonococcal urethritis (*Mycoplasma genitalium*). However, there is an interesting exception: *Mycoplasma alligatoris*, a pathogen of alligators, causes lethal infections. Although the infections caused by mollicutes are rarely lethal, mollicutes pathogenic for plants and animals cause a significant economic loss in agriculture. This is true for cattle in Africa that are infected by *Mycoplasma mycoides* as

Table 1 The systematic groups of the mollicutes

Order	***Genus***	***Genome size***	***Sterol requirement***	***Characteristics***	***Habitat***
Mycoplasmatales	*Mycoplasma*	580–1350 kb	Yes	Growth optimum: 37 °C UGA as Trp codon	Humans, animals
	Ureaplasma	760–1170 kb	Yes	Urea hydrolysis UGA as Trp codon	Humans, animals
Entomoplasmatales	*Entomoplasma*	790–1140 kb	Yes	Growth optimum: 30 °C	Insects, plants
	Mesoplasma	870–1100 kb	No	Growth optimum: 30 °C UGA as Trp codon	Insects, plants
	Spiroplasma	780–2200 kb	Yes	Growth optimum: 30–37 °C UGA as Trp codon Helical motile filaments	Insects, plants
Anaeroplasmatales	*Anaeroplasma*	1500–1600 kb	Yes	Obligate anaerobes	Bovine/ovine rumen
	Asteroleplasma	1500 kb	No	Obligate anaerobes	Bovine/ovine rumen
Acholeplasmatales	*Acholeplasma*	1500–1650 kb	No	Growth optimum: 30–37 °C UGA as stop codon	Animals, plants, insects
	Phytoplasma	640–1185 kb	Not known	Uncultured *in vitro* UGA as stop codon	Insects, plants

well as for rice crops in some regions of Southeast Asia that are infected by phytoplasmas. These losses not only have an economic dimension, but also a significant effect on human nutrition in the affected regions. *Mycoplasma* species such as *Mycoplasma hyorhinis* or *Acholeplasma laidlawii* are major sources of cell culture contamination and have gained increasing interest. These infections are often discovered only late in the course of an experiment and can invalidate the scientific research.

The close association of mollicutes with eukaryotic hosts and their adaptation to habitats with a good nutrient supply and relatively constant growth conditions led to a remarkable process of reductive genome evolution. The organism with the smallest known genome capable of independent life (if provided with rich artificial medium) is *M. genitalium*, a human pathogen. This organism has a genome size of only 580 kb and encodes about 480 proteins, as compared to about 4 million bp and 4000 genes for bacteria such as *Escherichia coli* or *Bacillus subtilis*. These small genomes made the mollicutes important tools for the new discipline of synthetic biology (see 'Genomic comparisons of mollicutes').

The Systematics of the Mollicutes

Evolution of the Mollicutes

The analysis and comparison of 16S rRNA sequences revealed that the mollicutes belong to the Gram-positive bacteria with genomes of low GC content. Ironically, most members of this phylum are characterized by their thick Gram-positive cell wall, and the group is therefore referred to as the firmicutes. This bacterial phylum includes the lactic acid bacteria (such as *Streptococcus* and *Lactobacillus*), spore-forming bacteria (*Bacillus* and *Clostridium*) and their close relatives (*Listeria* and *Staphylococcus*). As can be seen in the phylogenetic tree of the firmicutes (**Figure 1**), the mollicutes form a sister group to the large *Bacillus*/lactic acid bacteria group. It is believed that the first mollicutes emerged some 600 million years ago and that significant loss of ancestral genomic sequences was a major force in the evolution of the mollicutes.

The mollicutes are subdivided in several ways. Three traditional classifications rely on genetic or physiological properties of the bacteria, whereas more recent classification schemes are based on the similarity of the 16S rRNA or conserved protein families.

Two large groups of mollicutes can be distinguished based on their host organisms. Although most mollicutes infect exclusively animal hosts, there are other representatives (*Spiroplasma* and *Phytoplasma*) that are capable of infecting both plant and insect hosts. Another conventional way of classifying the mollicutes is based on their requirement for sterols. Most genera need sterols for growth, whereas this is not the case for the members of the genus *Acholeplasma* (see **Table 1**). However, this requirement can only be determined for those mollicutes that can be cultivated, and many (perhaps most) representatives have not yet been cultured, including all species of the genus *Phytoplasma*. Another peculiarity of most mollicutes is their codon usage: they use the UGA codon to specify tryptophan rather than as a stop codon as in the universal genetic code. Only the genera *Acholeplasma* and *Phytoplasma* among the mollicutes use UGA as a stop codon. Because this is the ancestral property, it can be assumed that *Acholeplasma* and *Phytoplasma* represent the more ancestral mollicutes. This

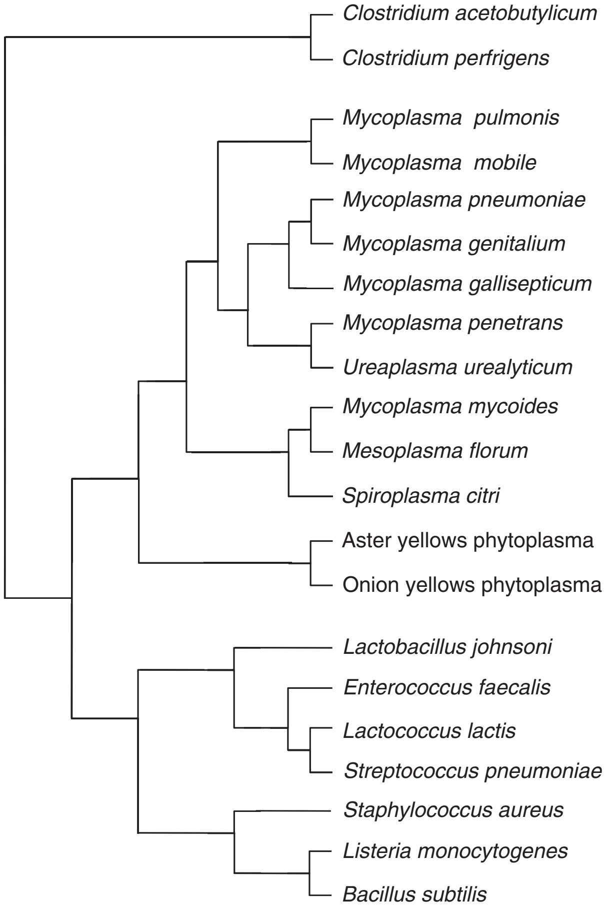
Clostridium acetobutylicum
Clostridium perfrigens
Mycoplasma pulmonis
Mycoplasma mobile
Mycoplasma pneumoniae
Mycoplasma genitalium
Mycoplasma gallisepticum
Mycoplasma penetrans
Ureaplasma urealyticum
Mycoplasma mycoides
Mesoplasma florum
Spiroplasma citri
Aster yellows phytoplasma
Onion yellows phytoplasma
Lactobacillus johnsoni
Enterococcus faecalis
Lactococcus lactis
Streptococcus pneumoniae
Staphylococcus aureus
Listeria monocytogenes
Bacillus subtilis

Figure 1 Unrooted phylogenetic tree of the firmicutes with special emphasis to the mollicutes. The tree is based on a concatenated alignment of 31 universal protein families. Reproduced from Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, and Bork P (2006) Toward automatic reconstruction of a highly resolved tree of life. *Science* 311: 1283–1287.

conclusion is supported by a phylogenetic tree based on a concatenated alignment of 30 protein families present in all mollicutes that places the genus *Phytoplasma* at the bottom of the tree (**Figure 1**). The genus *Acholeplasma* is not included in this analysis because of the lack of genome sequence information. It is interesting to note that the genus *Mycoplasma* is paraphyletic, and that genera such as *Spiroplasma*, *Mesoplasma*, and *Ureaplasma* have specific relatives among the different *Mycoplasma* clades (**Figure 1**).

For practical reasons, the mollicutes are grouped in four orders that do not represent the phylogenetic relationships. An overview of these taxa is provided in **Table 1**.

Mycoplasma

As mentioned earlier, the genus *Mycoplasma* is a paraphyletic collection of mollicutes that are widespread in nature as parasites of humans, mammals, birds, reptiles, and fish. The first representative of the genus *Mycoplasma* was identified in 1898 as the causative agent of contagious bovine pleuropneumonia (*M. mycoides*). The human pathogens *Mycoplasma hominis* and *M. pneumoniae* were discovered in 1937 and 1944, respectively. Even now, new species are being identified: in 1981, *M. genitalium* was isolated from a patient suffering from nongonococcal urethritis, and more recently, *Mycoplasma penetrans* and *Mycoplasma fermentans* were found to be associated with HIV infections.

The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, the eyes, the alimentary canal, and mammary glands. In addition, cell cultures are an artificial habitat for many *Mycoplasma* species. The mycoplasmas exhibit a rather strict host and tissue specificity, probably reflecting their highly specific metabolic demands and their parasitic lifestyle. For example, *M. pneumoniae* and *M. genitalium* are preferentially detected in the respiratory and urogenital tracts, respectively.

If cultivated in the laboratory, mycoplasmas as well as other mollicutes require complex media containing sugars, amino acids, nucleotides, and vitamins. It has so far been impossible to cultivate them on chemically defined media.

The complete genome sequences of ten species of the genus *Mycoplasma* have been determined so far. This large interest in the variability of the *Mycoplasma* genetic complement is stimulated by the interest in creating artificial organisms based on the *Mycoplasma* species (i.e., synthetic biology; see following text). The genome sequences revealed the reason for the complex nutritional requirements of the mycoplasmas: they lack the genes for many biosynthetic pathways and are thus dependent on their host or on the artificial medium to provide these required nutrients. Another interesting feature revealed by genome sequences is that only very few known regulatory proteins are present. Again, this is reflective of their close adaptation to one single natural habitat and a result of the reductive evolution: while a metabolically versatile bacterium such as *Pseudomonas aeruginosa* that is capable of thriving in a wide variety of environments reserves as much as 10% of its genome for regulatory genes, only a handful of these genes is found in the mycoplasmas (see following text).

Pathogenicity has been most intensively studied with *M. pneumoniae*. In contrast to most other pathogenic bacteria, *M. pneumoniae* and other mollicutes do not seem to produce any exo- or endotoxins. However, a recent study suggests the formation of a protein similar to ADP-ribosylating and vacuolating cytotoxin. However, this observation has not been confirmed by other groups. A major factor contributing to cytotoxicity and thus to pathogenicity of *M. pneumoniae* is the formation of hydrogen peroxide. The synthesis of hydrogen peroxide by mycoplasmas is most strongly increased if the bacteria are supplied with glycerol. This can be attributed to the oxidase activity of the enzyme that oxidizes glycerol

3-phosphate. This enzyme, glycerol-3-phosphate oxidase, uses water rather than NAD^+ (as in typical glycerol-3-phosphate dehydrogenases) as the electron acceptor. The hydrogen peroxide formed by *M. pneumoniae* acts in concert with endogenous toxic oxygen molecules generated by the host cells and induces oxidative stress in the respiratory epithelium. The effects of the peroxide on the host cells include loss of reduced glutathione, denaturation of hemoglobin, peroxidation of erythrocyte lipids, and eventually the lysis of the cells. Another result of infection by *M. pneumoniae* is the release of proinflammatory cytokines by the host cells. It has been suggested that cytokine production leads to chronic pulmonary diseases such as bronchial asthma.

The significance of glycerol metabolism in hydrogen peroxide production and virulence has been convincingly demonstrated by a series of studies that started with an analysis of the differences between European and African strains of *M. mycoides*, the causative agent of contagious bovine pleuropneumonia. Glycerol transport is highly efficient in the African isolates, whereas it is barely detectable in the European isolates. Because glycerol catabolism gives rise to the formation of hydrogen peroxide, it is not surprising that hydrogen peroxide production is high in the African strains but low in the European isolates of *M. mycoides*. In consequence, the African strains are highly virulent to cattle, whereas their European relatives are harmless. It has been hypothesized that intracellular formation of large quantities of hydrogen peroxide would be toxic for the producing cells themselves. Accordingly, the cellular localization of the responsible enzyme, GlpO, was studied in *M. mycoides* and it was found to be located in the cell membrane. The inactivation of GlpO by antibodies results in the loss of cytotoxicity of *M. mycoides* toward bovine epithelial cells. Given that hydrogen peroxide in concentrations similar to those produced by *M. mycoides* is not cytotoxic, it was concluded that GlpO is not only inserted in the bacterial cell membrane but also in the membrane of the host cell to inject the cytotoxic hydrogen peroxide directly into the epithelial cells. This may cause oxidative stress and subsequent cell death.

Plant Pathogenic Mollicutes: *Spiroplasma* and *Phytoplasma*

The genera *Spiroplasma* and *Phytoplasma* contain plant-pathogenic mollicutes that shuttle between plant and insect hosts. *Spiroplasma citri* was identified in 1971 as a causative agent of citrus stubborn disease. Phytoplasmas were first described in 1967 as the probable cause of plant yellow diseases. Originally, it was speculated that these diseases are of viral origin, and only in 1967 it became clear that these pathogens are *Mycoplasma*-like organisms. While spiroplasmas can be cultivated in the laboratory, no cultivation of any representative of the phytoplasmas has been reported. Therefore, no valid species description for members of the genus *Phytoplasma* is available. Moreover, *Spiroplasma* cells have a spiral morphology, whereas phytoplasmas are pleomorphic.

Spiroplasma species live in the phloem sieve tubes of their host plants. They are transmitted by insect vectors that feed on the phloem sap. Multiplication of the bacteria occurs both in the plant and in the insect hosts. The most intensively studied representative of the genus, *S. citri*, infects periwinkle (*Catharanthus roseus*) and its vector, the leafhopper *Circulifer haematoceps*. Unfortunately, no genome sequences of any *Spiroplasma* species are so far publicly available, although the *Spiroplasma kunkelii* genome has recently been sequenced.

The spiroplasmas are unique among the mollicutes for their helical cell morphology, and also by their unique mechanism of locomotion. The genetic determinants for this distinct morphology and movement are so far unknown. Although the spiroplasmas have a shape that is similar to that of the members of the genus *Spirillum*, they are different because they do not possess flagella. Propulsion is generated by a propagation of kink pairs down the length of the cell, caused by a processive change of cell helicity. In addition, these waves of kinks seem to be initiated always by the same end of the cell suggesting cell polarity. Cell polarity can also be concluded from the results of diverse microscopic studies that showed heterogeneity of both ends: one end is tapered with a tip-like structure called terminal organelle and the other one is blunt or round.

An interesting aspect of the *S. citri* lifecycle is the differential utilization of carbohydrates as source of carbon and energy in the two hosts. *S. citri* possesses the genetic equipment for the utilization of sorbitol, trehalose, glucose, and fructose as carbon sources, which are mainly catabolized to acetate. The two habitats of *S. citri* differ significantly in their carbon source availability. While glucose and fructose are predominant in phloem sieve tubes of plants, trehalose is the major sugar in the hemolymph of the vector insect, the leafhopper *C. haematoceps*. The glucose and trehalose permeases of the *S. citri* phosphotransferase system (PTS) share a common IIA domain encoded by the *crr* gene, which might be involved in the rapid physiological adaptation to changing carbon supplies. The glucose and fructose found in the plant sieve tubes are both derived from the cleavage of sucrose by the plant enzyme invertase. A transposon mutagenesis study with *S. citri* revealed that mutants devoid of a functional *fruR* gene encoding the transcriptional activator of the fructose utilization operon are no longer phytopathogenic. The fructose operon of *S. citri* contains three genes, *fruR*, *fruA*, and *fruK* encoding the transcription activator, the fructose-specific permease of the PTS, and the fructose-1-phosphate kinase, respectively.

Mutations in the *fruA* and *fruK* genes also resulted in decreased phytopathogenicity. However, these mutant strains could revert, and this reversion also restored severe symptoms upon plant infection. Thus, fructose utilization and pathogenicity are intimately linked in *S. citri*. In contrast to mutations affecting fructose utilization, a *ptsG* mutation abolishing glucose transport into the cell does not result in reduced pathogenicity of *S. citri*. The reason for the differential implication of the two sugars in pathogenicity was studied by nuclear magnetic resonance analysis and it turned out that the bacteria use fructose preferentially, whereas the glucose accumulated in the leaf cells of the infected plants. This led to the following model. In noninfected plants, both fructose and glucose are formed by invertase. Fructose inhibits this enzyme resulting in a very low activity. In contrast, no inhibition occurs in infected plants because of fructose utilization by *S. citri*. The accumulating glucose that is not used by the bacteria results in inhibition of photosynthesis and thus in the different symptoms.

Transmission from an infected plant to an insect vector occurs by the uptake of bacteria along with the phloem sap. Inside the leafhopper, the bacteria have to pass the intestine midgut lining to multiply in the hemolymph, and then infect the salivary glands. Infection of the salivary glands is important because transmission from the insect to a host plant occurs by inoculation of the saliva into the damaged plant during feeding. It was shown that certain adhesins are necessary for transmissibility of *S. citri* from an infected plant to a vector, and that the genes coding for these adhesins are located on plasmids not existing in all *S. citri* strains.

In contrast to the spiroplasmas whose members are pathogenic to a broad range of plants and insects, the phytoplasmas form their own group among the mollicutes that is strictly pathogenic to plants. Like the plant-pathogenic spiroplasmas, they inhabit the phloem sieve tubes of their host plants after infection by an insect vector (usually belonging to the family of Cicadelli), but they depend completely on their host and so far it has been impossible to cultivate them *in vitro*. However, the genome sequences of three members of this group, *Candidatus Phytoplasma asteris* onion yellows strain (OY-M), aster yellows *Phytoplasma* strain witches broom (AY-WB), and *Candidatus Phytoplasma australiense* have been determined.

Compared to other members of the mollicutes, the phytoplasmas have some unique features. They exhibit shapes that range from rounded pleomorphic cells, with an average diameter of 200–800 μm, to filaments. Their genomes lack all known genes coding for cytoskeleton or flagellum elements, suggesting that translocation of cells *in planta* is a passive event caused by the flow of phloem sap. As other mollicutes, the phytoplasmas lack genes for the *de novo* synthesis of amino acids, fatty acids, or nucleotides but they also lack some genes considered to be essential in all bacteria, such as *ftsZ* encoding a tubulinlike protein. As FtsZ is involved in cell division, the mechanism of division in the phytoplasmas lacking it must be completely different from that of other bacteria. Although living in an environment that is rich in carbon sources, neither of the sequenced phytoplasma possesses genes coding for sugar-specific components of the PTS. In contrast, *S. citri* and *S. kunkelii*, which thrive in the same environment as the phytoplasmas, contain three PTS for the import of glucose, fructose, and the insect-specific sugar trehalose (see earlier). However, *Phytoplasma* possesses the maltose-binding protein MalE. This protein may bind other sugars as well but genes for enzymes making these sugars available for glycolysis are absent. Sucrose, the main sugar in the phloem sap of plants, could be used as a source of carbon and energy, but in sequenced phytoplasmas the gene for sucrose phosphorylase, which is important for sucrose degradation, is absent or fragmented. In general, phytoplasmas possess fewer genes related to carbon metabolism than the other mollicutes. Energy generation in phytoplasmas seems to be restricted to glycolysis because ATP synthases are absent. OY-M *Phytoplasma* contains a P_{2C}-ATPase, which is common in eukaryotic cells but unique among prokaryotes. Another remarkable feature that makes the phytoplasmas unique among the mollicutes is their ability to synthesize phospholipids, supporting a closer phylogenetic relationship to *Acholeplasma*, which do not require sterols.

Biochemistry of the Mollicutes

Cytology of the Mollicutes

The mollicutes differ from other bacteria not only because they lack a cell wall but also by dint of their small cell sizes. A typical cell of *M. pneumoniae* is 1–2 μm long and 0.1–0.2 μm wide (**Figure 2**). In contrast, a typical rod-shaped bacterial cell (such as *E. coli* or *B. subtilis*) is 1–4 μm in length and 0.5–1 μm in diameter.

The absence of a cell wall has serious consequences for the osmotic stability of the mollicute cells. They are much more sensitive to changes of the osmotic conditions than bacteria possessing a cell wall. The parasitic lifestyle of the mollicutes may be directly related to their osmotic sensitivity: the hosts provide them with osmotically constant conditions that would not be found in the external environment. For example, *M. genitalium* is a parasite of the human urogenital tract, and its transmission by sexual contact ensures minimal exposure of the bacteria to an external, osmotically variable, environment. With the exception of the phytoplasmas and acholeplasmas, the mollicutes are unable to produce fatty acids for membrane biosynthesis and are therefore dependent on exogenously provided fatty acids, which are then used

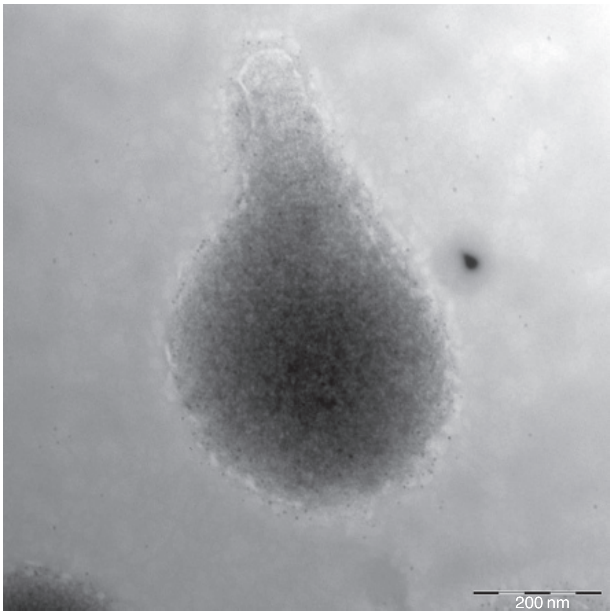

Figure 2 Electron micrograph of a cell of *Mycoplasma pneumoniae*. The terminal organelle (also called the tip structure) is visible in the upper part of the cell. Scale bar = 200 nm.

for phospholipid synthesis. The lack of fatty acid synthesis is accompanied by the absence of a fatty acid desaturase, which is required to adapt the membrane fluidity to lower temperatures. To overcome this difficulty, most mollicutes incorporate large amounts of sterols, which serve as a very effective buffer of membrane fluidity (see **Table 1**).

The lack of a cell wall has also consequences for the cellular morphology of the mollicutes. The cells are pleomorphic; however, they are not small amoebas! The mollicutes exhibit a variety of morphologies, such as pear-shaped cells, flask-shaped cells with terminal tip structures (see below), filaments of various lengths, and in the case of *Spiroplasma* species the cells are helical.

The mycoplasmas have a flask- or clublike shape with a terminal organelle, the so-called tip structure (see **Figure 2**). This tip structure is a complex and specialized attachment organelle that has evolved to facilitate the parasitic existence of the mycoplasmas. The tip structure is made up of a network of adhesins, interactive proteins, and adherence accessory proteins, which cooperate structurally and functionally to mobilize and concentrate adhesins at the tip of the cell. The major adhesin of *M. pneumoniae* is the 170 kDa P1 protein that is responsible for the interaction of the bacteria with the host cells. In addition, the tip structure is important for the internalization of intracellular mollicutes such as *M. penetrans* and *M. genitalium*. *M. penetrans* is capable of actively entering different types of animal cells, even those with minimal phagocytic activity. This may protect the bacterial cells against the host immune system. The formation of the tip structure in *M. pneumoniae* depends on the activity of the P41 protein that serves as an anchor protein. In the absence of this protein, multiple terminal organelles form at lateral sites of the cell and the terminal organelles are not attached to the body of the cell. In *Mycoplasma mobile*, there is also a terminal structure that is referred to as the 'jellyfish' structure made up of a 'bell' with dozens of flexible tentacles. Several components of this structure have been identified. With the exception of the glycolytic enzyme phosphoglycerate kinase, these *M. mobile* proteins are all absent from the genome of *M. pneumoniae* suggesting that the two species found individual solutions for the assembly of the terminal organelle.

Mycoplasma species are able to glide on solid surfaces with the help of their terminal attachment organelle. Terminal organelles that are detached from the body of the *M. pneumoniae* cell are released by some mutants. These detached organelles are still capable of gliding demonstrating that this organelle acts as a novel engine that allows cellular movement. The fastest gliding *Mycoplasma* species, *M. mobile*, contains a dedicated 349 kDa 'leg' protein that is required for gliding. This protein is composed of an oval base with three successive flexible extensions that may support movement. Movement is thought to occur by repeated catching and releasing of sialic acid on solid surfaces and is driven by the hydrolysis of ATP. This ATP hydrolysis may be catalyzed by the glycolytic enzyme phosphoglycerate kinase that is part of the terminal organelle in *M. mobile*.

As other bacteria, the mollicutes divide by binary fission. Again, the terminal organelle seems to be very important for this process: Cell division in *M. pneumoniae* is preceded by the formation of a second tip structure adjacent to the existing one. The two terminal organelles then separate leading eventually to cytokinesis. Among the proteins known to be important for bacterial cell division is the tubulinlike GTP-hydrolyzing FtsZ protein that forms a ring at the division site. Until recently, FtsZ proteins were found in any newly analyzed genome, and the *ftsZ* gene is essential in most bacteria, including *E. coli* and *B. subtilis*. Therefore, FtsZ was considered to be indispensable for all life. However, it recently turned out that some mollicutes such as *M. mobile, Ureaplasma urealyticum*, and the two sequenced phytoplasmas lack *ftsZ* genes, suggesting that its function is dispensable at least in some mollicutes. In many bacteria, the FtsA protein is required for the recruitment of the proteins that form the septum for cell division. Interestingly, this protein is absent from all the pleomorphic mollicutes, whereas it has been detected in *S. kunkelii*. This may be related to the helical morphology of these bacteria.

Metabolism of the Mollicutes

The reductive evolution of the mollicutes is reflected in their limited metabolic properties. Of the central metabolic pathways, that is, glycolysis, the pentose phosphate shunt, and the tricarboxylic acid (TCA) cycle, only glycolysis seems to be operative in most mollicutes. Most striking is the lack of many energy-yielding systems in the mollicutes. No quinones or cytochromes were found in any representative. The electron transport system is flavin-terminated. Thus, ATP is produced by substrate-level phosphorylation, a less efficient mechanism as compared to oxidative phosphorylation.

As observed for *M. genitalium* glyceraldehyde 3-phosphate dehydrogenase, the glycolytic kinases of several mollicute species have functions in addition to that in glycolysis. These enzymes can use not only ADP/ATP but also other nucleoside diphosphate/triphosphate couples. Thus, these enzymes (phosphofructokinase, phosphoglycerate kinase, pyruvate kinase, and acetate kinase) compensate for the lack of the normally essential *ndk* gene encoding nucleoside diphosphate kinase that is required for nucleotide biosynthesis.

Glycolysis is not the only source of ATP formation by substrate level phosphorylation in the mollicutes. Pyruvate can be oxidized to acetyl-CoA by pyruvate dehydrogenase. Acetyl-CoA can be further catabolized by phosphotransacetylase and acetate kinase in an additional substrate level phosphorylation resulting in the formation of acetate. An alternative pathway of pyruvate consumption is its reduction to lactate, leading to the regeneration of NAD^+.

A recent study with *M. pneumoniae* demonstrated that glucose is the carbon source allowing the fastest growth of these bacteria. In addition, *M. pneumoniae* can utilize glycerol and fructose. Interestingly, mannitol is not used even though the genetic equipment to utilize this carbohydrate seems to be complete. Obviously, one or more of the required genes are not expressed or inactive.

Glucose and fructose are transported into the cells by the PTS. This system is made up of general soluble components and sugar-specific membrane-bound permeases. The general components, enzyme I and HPr, transfer a phosphate group from phosphoenolpyruvate to the sugar permease, which phosphorylates the sugar concomitant to its transport.

The arginine dihydrolase pathway can be found also in some *Spiroplasma* and *Mycoplasma* species. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2, and ammonia. The pathway uses three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase. The degradation of arginine is coupled to equimolar generation of ATP by substrate-level phosphorylation. The role of this pathway as a sole energy-generating source in mycoplasmas is questionable. However, the existence of an arginine–ornithine antiport system in *Spiroplasma melliferum* requiring no ATP for arginine import into the cells supports an energetic advantage in arginine utilization.

Mollicutes possess very limited metabolic and biosynthetic activities for amino acids, carbohydrates, and lipids as compared to ‘conventional’ bacteria. *M. pneumoniae* scavenges nucleic acid precursors and does not synthesize purines or pyrimidines *de novo*. These may be provided by RNA and DNA that have been degraded by potent mycoplasmal nucleases. Furthermore, both *M. genitalium* and *M. pneumoniae* lack all the genes involved in amino acid synthesis, making them totally dependent on the exogenous supply of amino acids from the host or from the artificial culture medium. The mycoplasmas have also lost most of the genes involved in cofactor biosynthesis; therefore, to cultivate them *in vitro*, the medium has to be supplemented with essentially all the vitamins.

Being dependent on the exogenous supply of many nutrients would predict that mycoplasmas need many transport systems. Surprisingly, *M. genitalium* and *M. pneumoniae* possess a only small number of transport proteins (34 and 44 proteins, respectively) compared to the 281 transport and binding proteins annotated in *E. coli* and almost 400 in *B. subtilis*. The apparent low substrate specificity of some of the mollicute transport systems, such as those for amino acids, may also contribute to the significant gene reduction observed.

Although mollicutes produce hydrogen peroxide, *M. pneumoniae* and *M. genitalium* lack the genes dealing with oxidative stress, such as those encoding catalase, peroxidase, and superoxide dismutase. A thioredoxin reductase system, identified in the mycoplasmas, may protect them from reactive oxygen compounds.

A major problem for the research with mollicutes is the difficulty of cultivating them *in vitro*. Only a minority of the mollicutes existing in nature have been cultivated so far. For example, none of the phytoplasmas infecting insects or plants has been cultivated *in vitro*. To overcome the metabolic deficiencies of the mycoplasmas, complex media are used for their cultivation. The media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplements. Serum has been shown to provide, among other nutrients, fatty acids and sterols that are required for membrane synthesis. The requirement for sterols has served as an important taxonomic criterion distinguishing the sterol-nonrequiring mycoplasmas, particularly the *Acholeplasma* species, from the sterol-requiring ones. For most mycoplasmas, the pH is adjusted to a slightly alkaline value, conditions that imitate those in the eukaryotic host. A common approach to improve *in vitro* cultivation of fastidious mycoplasmas is based on coculture with eukaryotic cell lines (cell-assisted growth). In this way, some spiroplasmas, such as the Colorado potato beetle *Spiroplasma*, were first successfully cocultivated with insect cell lines.

Genetics and Molecular Biology of the Mollicutes

Gene Expression in the Mollicutes

The basic mechanisms of gene expression have been studied poorly in the mollicutes. They possess a conventional bacterial RNA polymerase, but unlike most other bacteria, they encode only one sigma factor of the RNA polymerase. Thus, diversity of promoters and RNA polymerase holoenzymes are not used for regulatory purposes in the mollicutes. The transcription start sites have been identified for several *M. pneumoniae* genes, and it turned out that the −10 region of these promoters is similar to that recognized by the housekeeping sigma factors of other bacteria such as *E. coli* or *B. subtilis*. In contrast, there is no conserved −35 region. These observations were confirmed by a recent analysis of the sequence determinants that are required for promoter activity in front of the *M. pneumoniae ldh* gene encoding lactate dehydrogenase. The −10 region is essential for transcription initiation, whereas the −35 region could be mutated without any consequences. Thus, the single *M. pneumoniae* RNA polymerase holoenzyme recognizes only the −10 region for promoter recognition.

Another peculiarity of the *M. pneumoniae* transcription machinery is the lack of the termination factor Rho, and correspondingly, the absence of Rho-dependent transcription terminators. Surprisingly, a bioinformatic analysis of bacterial genomes and the free energy values of RNAs around the end of open reading frames suggest that the mollicutes do also not contain functional Rho-independent transcription terminators. This raises the important question of how transcription is terminated in the mollicutes or whether it is terminated at all. The answer came from Northern blot experiments aimed at the identification of *in vivo* transcripts, and this answer is ambiguous. Indeed, defined transcripts were observed in a few cases, such as the *M. genitalium* and *M. pneumoniae ftsZ* gene clusters or the *M. pneumoniae ptsH* gene. The existence of these defined transcripts implies that there are also defined transcription terminators present. However, these terminators may be very rare. This might explain the observation that unrelated genes are expressed as parts of one transcription unit in the mollicutes. Moreover, most attempts to determine transcript sizes by Northern blot analysis in the mollicutes have failed. This is probably the result of mRNA length polymorphisms, which prevent the detection of clearly defined RNA species.

Most genes in the mollicutes have the same orientation on the chromosome, and the intergenic regions are usually quite short if present at all. The transcription of most of these large gene clusters is colinear with replication. This genome organization also favors polycistronic transcription of large gene clusters.

The lack of defined mRNA species results not only from the absence of transcription terminators but also from the weak conservation of sequences that mediate transcription initiation: a −10 region made up of only Ts and As is statistically overrepresented in the AT-rich mollicute genome. Indeed, the −10 regions predicted from the analysis of many start points occur about 2900 times in the 816 kb genome of *M. pneumoniae*. This large number of possible transcription initiation sites is also reflected by the observation of substantial antisense transcription in both *M. genitalium* and *M. pneumoniae*.

In bacteria, regulation is usually exerted at the level of transcription. In the mollicutes, only one example of transcription regulation is clearly documented: this is the regulation of the *S. citri* fructose operon by the transcription activator FruR (see earlier text). Moreover, the induction of chaperone-encoding genes at elevated temperatures was demonstrated in several *Mycoplasma* species. By analogy to the mechanism of heat shock regulation by the repressor protein HrcA and the DNA operator element CIRCE, it was proposed that heat shock genes are under the control of HrcA in the mollicutes. In addition to HrcA, the genomes of *M. genitalium* and *M. pneumoniae* encode only two other potential transcription factors that belong to the GntR and the Fur family, respectively. Unfortunately, the function of these regulators has so far not been studied.

It is interesting to note that *M. pneumoniae* contains only three potential regulators (less than 0.5% of all open reading frames), whereas environmental bacteria such as *Streptomyces coelicolor* and *P. aeruginosa* reserve about 10% of their genetic capacity to encode transcription factors. The low number of transcription factors in the mollicutes and the weak stringency of transcription signals in the mollicutes might therefore reflect their close adaptation to specific habitats that provide a good supply of nutrients and protect the bacteria from harmful environmental conditions. Moreover, the good supply of nutrients from external sources, that is, the host, may abolish the need for transcription regulation, that is, to switch off the expression of genes if their products are not required.

An additional mechanism of regulation is provided by riboswitches and regulatory RNAs. A guanine-specific riboswitch was detected in the untranslated region of the *Mesoplasma florum guaAB* operon suggesting that this RNA element governs the regulation of this operon via guanine.

Translation is one of the most prominent activities of the mollicute cell: as much as 15% of the genome of the mollicutes is devoted to translation-related functions. The principal mechanisms of translation in the mollicutes are identical to those found in other bacteria. Because of the low genomic GC content, the codon usage is strongly biased toward AT-rich codons. With the exception of

Phytoplasma and *Acholeplasma*, the mollicutes decode the UGA codon as tryptophan instead of using it as a stop codon as in the universal genetic code. This poses severe problems for the expression of mollicute proteins in heterologous hosts (see following text).

The mechanisms of translation initiation seem to differ among the mollicutes. In some organisms such as *Mycoplasma capricolum* and *S. citri*, the open reading frames are preceded by canonical Shine–Dalgarno sequences that form base pairs with the 3′ end of the 16S rRNA. In contrast, many genes of *M. pneumoniae* and *M. genitalium* lack such a sequence, and moreover, leaderless mRNAs are common in these bacteria. The molecular mechanisms of translation initiation in *M. pneumoniae* and its close relatives still await elucidation.

Posttranslational Protein Modification

In many bacteria including the mycoplasmas, the HPr protein of the PTS cannot only be phosphorylated by enzyme I but is also the target of a regulatory phosphorylation on Ser-46 by a metabolite-activated protein kinase, HPrK. The phosphorylation of HPr on Ser-46 in 'less degenerated' firmicutes leads to carbon catabolite repression. So far, the functions of HPrK and ATP-dependent phosphorylation of HPr have not been studied in the mollicutes. In contrast, much work has been devoted to the biochemical characterization of HPrK from *M. pneumoniae*. Unlike its equivalent from other bacteria, this protein is active at very low ATP concentrations. As in related proteins, it contains an essential Walker A motif for ATP binding. Mutations in this region severely affect both the kinase and the phosphatase activities of the protein. Fluorescence studies revealed that the *M. pneumoniae* HPrK has a significantly higher affinity for ATP than any other HPrK studied so far. This may explain why it is active even at low ATP concentrations. The *M. pneumoniae* HPrK was crystallized and its structure determined. As observed for homologous proteins, it forms a hexamer with the C-terminal domains in the active center.

In addition to HPrK, there is one other protein kinase in *M. pneumoniae* and many other mollicutes, PrkC. The corresponding gene is clustered with the gene encoding a protein phosphatase of the PP2C family, PrpC. It was shown that PrpC is implicated in the dephosphorylation of HPr(Ser-P). PrkC is known to phosphorylate a wide variety of proteins in other firmicutes; however, its targets and the role of PrkC-dependent phosphorylation in the mollicutes remain to be studied.

Protein phosphorylation seems to be important for the biology of the mollicutes. An analysis of the *M. genitalium* proteome revealed that each identified protein is present at an average of 1.22 spots on a 2-D gel, suggesting posttranslational modification of about 25% of all proteins. Given the importance of protein phosphorylation in all other living organisms, it seems safe to assume that a large portion of these modified proteins is actually phosphorylated. A phosphoproteome analysis of *M. genitalium* and *M. pneumoniae* identified 5 and 3% of the total protein complement of these bacteria, respectively, as phosphoproteins. Among these proteins are not only enzymes of central carbon metabolism such as enolase and pyruvate dehydrogenase subunits, but also several cytoskeleton and cytadherence proteins. It is tempting to speculate that PrkC may catalyze these phosphorylation events.

As in other bacteria, there is protein secretion in the mollicutes. While some exported proteins carry typical signal peptides at their N-termini, there is no signal peptidase I present in the genome of the mollicutes. This raises the possibility that so far uncharacterized proteins are active in protein secretion in the mollicutes.

Genomic Comparisons of Mollicutes

One of the questions that have been of interest to humans since its early days is the problem of what constitutes life. Only today, in the era of genome research, are we able to attempt an answer to this question. A major milestone in defining life was the identification of key features that characterize all living things and differentiate them from nonliving matter such as viruses and prions. Among these features are metabolism, autonomous replication, communication, and evolution. With the availability of genome sequences, it is has become possible to determine the genetic equipment required for independent life. The mollicutes are of special interest in this respect because they have the smallest genomes that allow independent life, at least under laboratory conditions.

Genome research with the mollicutes is driven by two major challenges: (1) the identification of the minimal set of genes that is required for independent life and (2) the creation of artificial organisms that are based on this minimal gene set. The simplicity of the mollicutes and the broad body of knowledge on their biology makes them ideal starting points for these research areas.

Several different strategies have been applied to identify the minimal gene set required for life. The most simple approach is based on the comparison of sequenced genomes of different organisms. It seems safe to assume that those genes that are conserved in different organisms are more important than those that appear only in certain species. The smallest genome of any independent living organism known so far is that of *M. genitalium*. This bacterium has a genome of 580 kb with 482 protein-coding genes and 39 genes coding for RNAs. *M. pneumoniae* has a genome of 816 kb with 779 genes coding for proteins and 40 RNA-coding genes. A comparison of the two genomes reveals an overlap of 477 genes common to both species. This suggests that *M. pneumoniae* is an 'extended version' of *M. genitalium*. It is tempting to speculate that *M. genitalium* is further advanced on the pathway of reductive genome evolution. Indeed, some genes present in *M. pneumoniae* but not in *M. genitalium* such as the mannitol utilization genes are known to be nonfunctional in the former organism. Thus, *M. genitalium* seems to be very close to a true minimal organism.

A comparison of all sequenced mollicute genomes reveals that only a small subset of their genes is part of a common gene pool. Only 156 genes are common to all mollicute genomes that have so far been sequenced. This represents about one-third of the 482 open reading frames of *M. genitalium.* Interestingly, of the 156 genes of the mollicute core genome, the large majority, that is, 124 genes, are shared by all firmicutes. Thus, there is only a small set of 32 genes that is conserved in all mollicutes but not in all firmicutes. However, even these genes are shared by many members of the firmicutes thus precluding the idea of a gene set unique to the mollicutes. Moreover, a large fraction of the common mollicute gene set forms the core genome of all bacteria (about 100 genes). Thus, the genome reduction of the mollicutes obviously went down to a minimum that is absolutely required for cellular life. This is becoming clear if one takes into account that even unrelated bacteria such as *E. coli* (γ-proteobacterium) and *B. subtilis* (firmicute) share about 1000 genes.

The core gene set of the mollicutes is made up mainly of genes encoding proteins involved in essential cellular functions such as DNA topology, replication and repair, transcription, RNA modification and degradation, translation, protein folding, secretion, modification, or degradation (**Table 2**). In addition, seven genes encoding potential GTP-binding proteins are conserved in all mollicute genomes. A few conserved metabolic genes encode proteins involved in glycolysis, metabolite and ion transport, nucleotide, lipid, phosphate, and amino acid metabolism. Interestingly, not a single protein of completely unknown function is conserved among all mollicutes. Moreover, the genes common to all mollicutes act in the central processes of life. This implies that there are no genes common to all mollicutes that are required for mollicute-specific activities such as the formation of the terminal organelle. This is in good agreement with earlier studies that demonstrated a large variability in the protein composition of this organelle.

A second approach to determine the minimal gene set required for life uses an experimental setup. Global transposon mutagenesis studies with *M. genitalium* and *M. pneumoniae* revealed dispensable genes. For *M. genitalium*, about 100 genes could be disrupted. This implies that the remaining 382 genes are essential. In addition, five genes that are part of groups of redundant genes seem to be essential. It is believed that these 387 genes (plus the RNA-coding genes) constitute the essential gene set of *M. genitalium.* The difference between the 156 genes in the core gene set of the mollicutes and the 387 genes that are essential for *M. genitalium* suggests that many of the additional genes are important under the specific ecological conditions of *M. genitalium.* This idea is supported by the presence of 110 genes of unknown function among the essential genes. This finding clearly demonstrates how much remains to be learned about the biology of *M. genitalium*, and surely about the other mollicutes as well.

With information on the minimal gene set in hand, the logical next step will be to construct artificial organisms with this set of genes. In 2007 and 2008, two important technological steps have been made on the way to the construction of such minimal artificial life: first, the replacement of one genome by another, a process called genome transplantation, was demonstrated. Genomic DNA of *M. mycoides* large colony (LC) was used to replace the genome of *M. capricolum* by polyethylene glycol-mediated transformation. The second major achievement was the chemical synthesis and assembly of the *M. genitalium* chromosome. Thus, an artificial chromosome can be synthesized and this DNA can be introduced into a living cell to provide the environment for the expression of this genome. The generation of an artificial minimal *Mycoplasma*-derived organism ('*Mycoplasma laboratorium*') would be the logical

Table 2 The core gene set of the mollicutes

Function	*Number of genes*
Information pathways—Protein	
Ribosomal proteins	38
Translation factors	11
Amino acyl tRNA synthetases	19
Chaperones	2
Proteolysis	3
Protein modification	1
Protein secretion	5
Information pathways—RNA	7
Transcription	7
RNA modification	8
RNA degradation and maturation	5
Information pathways—DNA	
Replication	7
Repair	8
DNA topology	3
Metabolism	
Basic carbon and energy metabolism	8
Amino acid metabolism	1
Nucleotide biosynthesis	6
Pyrophosphatase	1
Lipid metabolism	1
Miscellaneous functions	
Transport	7
GTP-binding proteins	7
Unknown proteins	7 (mge_009, 056, 132, 222, 366, 505, 516)

next step and the ultimate proof of both these technologies and of our understanding for the minimal equipment of a living cell.

Molecular Biology and Genetic Tools for the Mollicutes

The detailed genetic analysis of the mollicutes has been hampered for a long time by the lack of genetic tools that allow the efficient expression of UGA-containing mollicute genes in heterologous hosts for purification and subsequent biochemical analysis, the stable introduction of foreign genetic material into a mollicute cell, and either the targeted construction or the targeted isolation of desired mutant strains. During the past few years considerable progress has been made in the field of mollicute genetics, making these organisms accessible for genetic studies.

The occurrence of UGA codons in the genes of mollicutes has often prevented their expression in heterologous hosts for detailed biochemical analysis, because they serve as stop codons in *E. coli* and other expression hosts. To circumvent this problem, a variety of different but rather dissatisfying strategies had been employed, including the expression of UGA-containing genes in opal suppressor strains of *E. coli*, or in *S. citri* that also reads UGA as a tryptophan codon. As long as only few UGA codons are present in a gene, their sequential replacement by standard site-directed mutagenesis strategies might also be taken into consideration. However, the latter approach is time-consuming and cost-intensive with an increasing number of UGA codons. Recently, a strategy referred to as multiple mutation reaction (MMR) allowing the simultaneous replacement of multiple UGA codons in a single-step reaction was developed. This strategy is based on the use of 5′-phosphorylated oligonucleotides containing the desired mutations in a polymerase chain reaction (PCR). During the elongation steps, the external amplification primers are extended. As the mutation primers are designed to hybridize more strongly to their targets, the elongated amplification primers can then be ligated to the 5′ ends of the mutation primer by a thermostable DNA ligase, yielding a DNA strand that contains the desired mutation. With this strategy, the simultaneous introduction of up to nine mutations in one single step is possible.

The majority of genetic tools that are well established in model organisms are unavailable for mollicutes. Therefore, transposons are in common use for a variety of purposes. In combination with smart screening systems, they were used for the disruption of genes but also as carriers for the introduction of genetic material into the chromosome. The transposons Tn*916* and Tn*4001* and their improved derivatives can be used in mollicutes. These transposons were originally isolated from *Enterococcus faecalis* and *Staphylococcus aureus*, respectively, and have a broad host range. Tn*916* is a conjugative 18 kb transposable element that contains the *xis-Tn*/*int-Tn* genes for excision/integration, followed by the *tetM* tetracycline resistance determinant and a set of genes (*tra*) required for intercellular transfer. Tn*916* does not generate target duplications at its integration site, because it transposes by an excision/integration mechanism that is based on staggered nicks in the donor DNA. Tn*4001* is a 4.5 kb composite transposon consisting of two identical IS*256* elements flanking the gentamicin/kanamycin/tobramycin resistance conferring *aac-aphD* gene. Tn*4001* has been used for transforming several *Mycoplasma* species. To increase the stability of transposon insertion mutants, mini-transposons on the basis of Tn*4001* were constructed that have the transposase gene outside the transposable elements to prevent reexcision of the transposon after the first transposition event.

Until very recently, the targeted construction of gene knockout mutants via homologous recombination has only been reported in a few mollicutes such as *M. genitalium*, *Mycoplasma gallisepticum*, *Mycoplasma pulmonis*, and *A. laidlawii*. In the absence of homologous recombination, the only remaining way to obtain gene knockouts is transposon mutagenesis. Because of the randomness of integration, the screening of large transposon mutant libraries for the loss or gain of a specific phenotype is required to isolate a gene knockout of interest. If no screenable phenotype can be expected to be associated with a gene of interest, the only known feature of the desired gene knockout is the specific DNA junction between the gene of interest and the transposon. Based on this idea, a strategy referred to as 'haystack mutagenesis' has been designed that allows the targeted isolation of any viable transposon insertion strain out of an ordered library of transposon mutants. The concept of haystack mutagenesis is based on a saturating transposon mutagenesis to ensure that each dispensable gene is disrupted at a desired confidence level. Once the required number of transposon mutants has been isolated, they are arranged in pools of a reasonable size. These pools can then be screened by PCR using a gene-specific oligonucleotide and another one specific to the transposon for identifying the pool that contains the desired insertion. Subsequently, a similar screen at the level of the individual clones of the positive pool will identify the mutant of interest. This strategy has already been used for the isolation of several *M. pneumoniae* mutants. Alternatively, transposon mutant libraries can be screened for mutants that exhibit an interesting phenotype, such as loss of gliding motility.

The use of transposons is accompanied by the problem of changes of the genetic context at the site of integration that may cause undesired side effects. To avoid this problem, autonomously replicating plasmids have always been the vehicle of choice. Some early studies reported the isolation of naturally occurring plasmids from *M. mycoides*. These are small cryptic plasmids with a size in the range of 1.7–1.9 kb coding for replication functions only. Based on one of these plasmids, *M. mycoides–E. coli* shuttle vectors were developed. Further developments of artificial plasmid vectors were stimulated, when the first genome sequences became available that allowed the determination of the origins of replication of *Mycoplasma* chromosomes. Plasmid replicons have been constructed that contain the *oriC* sequences from *M. mycoides*, *M. capricolum*, and *Mycoplasma agalactiae*. Remarkably, a certain host specificity was observed for *oriC* plasmids, hampering the prediction the *oriC* compatibility between different *Mycoplasma* species and the derived plasmids. Nevertheless, with the genome sequence of many mycoplasmas at hand, the construction of stably replicating *oriC* plasmids for any desired *Mycoplasma* can be expected in the near future.

In the past there have been a couple of studies aimed at the definition of mycoplasmal promoters. The lack of clarity concerning the nature of gene expression/regulation signals in mollicutes (see 'Gene expression in the mollicutes') can only be answered in experiments that make use of promoter reporter systems. Such reporter systems based on the promoterless *lacZ* gene or on fluorescent proteins have been developed and used. They are used in two ways: the reporter genes can be randomly introduced into the chromosome to isolate random fusions with promoters; alternatively, the fusions can be prepared on plasmid vectors before their introduction into the genome. This second possibility allows the analysis of mutant promoter variants.

At present all required tools for the application of standard genetics to mycoplasmas are available. The biochemical *in vitro* analysis of individual proteins is no longer hampered by the genetic code of these organisms. Thus, interesting proteins can be easily studied. Similarly, antigenic surface proteins, which are often very large and thus contain many UGA codons can now easily be produced in heterologous hosts in sufficient amounts to be tested as vaccine candidates. Using the existing reporter systems, it will be possible to refine the mycoplasmal promoter concept, to discover regulatory DNA sequences and, ultimately, unravel the signal transduction mechanisms that mediate the adaptive responses seen in a wide variety of DNA microarray analyses but which are not yet understood at the molecular level. To confirm *in vitro* findings with purified proteins, targeted disruption of desired genes can presently be carried out in various representatives of the genus *Mycoplasma*, either by homologous recombination or by facilitated screening methods such as haystack mutagenesis.

Accompanying Feature

Additional resources on the mollicutes (key references, genome information, labs working on the mollicutes, information on important methods) can be found on an accompanying web page (http://tinyurl.com/3vw8ca).

Further Reading

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Nutrition, Microbial

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Defining Statement
Classification of Microorganisms and Nutrients
Elemental Composition of Biomass
Requirements and Physiological Functions of Principal Elements
Feast and Famine: Unrestricted versus Nutrient-Limited Growth
Design and Analysis of Defined Minimal Growth Media
Further Reading

Glossary

anabolism The process of synthesis of cell components from a metabolic pool of precursor compounds.

assimilation The incorporation of a compound into biomass.

catabolism The breakdown of nutrients to precursor compounds for anabolism or for dissimilation.

chemoautotrophy The use of reduced inorganic compounds and CO_2 as the primary sources of energy and carbon for biosynthesis.

chemoheterotrophy The process in which organisms are using organic compounds as the primary sources of carbon and energy for biosynthesis.

dissimilation The oxidation of a reduced (in)organic compound to provide energy for biosynthesis and cell maintenance.

growth medium An aqueous solution containing all the nutrients necessary for microbial growth.

limitation of growth The restriction on microbial growth by the availability of the nutrient that is first consumed to completion even when all other essential nutrients are present in excess. This growth-limiting nutrient determines the maximum amount of biomass that can be formed in this system; at low concentrations in batch culture and in the chemostat it also determines the rate (kinetics) of growth.

nutrient An organic or inorganic compound that is used by microorganisms as a building block for the synthesis of new cell material. In a wider sense, also compounds not incorporated into the microorganism, but serving as a source of energy or as terminal electron acceptor. Nutrients are grouped into classes depending on the physiological purpose they serve, the quantity required, and whether or not they are essential for growth.

nutritional categories of microorganisms Categories based on the principal carbon (CO_2 or reduced organic compounds) and energy sources (light or reduced (in)organic compounds) of microorganisms; there are four nutritional categories: photoautotrophs, photoheterotrophs, chemoautotrophs, and chemoheterotrophs.

photoautotrophy The use of light and CO_2 as the primary sources of energy and carbon for biosynthesis.

photoheterotrophy The use of light and reduced organic compounds as the primary sources of energy and carbon for biosynthesis.

Stoichiometry The relationship between the relative quantities of consumed nutrient and biomass formed during microbial growth; reflected in the growth yield coefficient (Y).

Abbreviations

AOC	assimilable organic carbon
EDTA	ethylene diamine tetraacetic acid
LB	Lysogeny broth
NTA	Nitrilotriacetic acid
PHAs	poly(3-hydroxyalkanates)
PHB	Polyhydroxybutyrate

Defining Statement

To grow and divide, microbial cells take up precursors and building blocks (nutrients) from the environment. In a wider sense, nutrients are also compounds that are not directly incorporated into cell material but are used by microbes to obtain the energy necessary to drive this synthesis and maintain cell integrity. Different nutritional types of microorganisms exist using different forms of carbon (CO_2 or reduced organic compounds) and energy (light or chemical energy) as the primary sources for biosynthesis. Nevertheless, the cellular composition of all microbial cells with respect to bulk components and the elemental composition is rather similar. Because of this, it is possible to estimate the general requirement of different nutrients for growth and to design and analyze microbial growth media. In well-designed growth media a particular identified nutrient is growth-limiting and determines the amount of biomass that can be formed, whereas all other nutrients are present in excess (Liebig's principle). Cell metabolism and performance are strongly influenced by the nature of the growth-limiting nutrient. Therefore, many industrial fermentation processes are based on restricting the availability of a particular nutrient in order to force a strain into a physiological state favorable for production.

Classification of Microorganisms and Nutrients

Growth and production of offspring is the ultimate goal of each microbial cell and to achieve this it takes up nutrients from the environment for two purposes: Either to serve as a source of building blocks or precursors for the synthesis of new cellular constituents, or to generate energy to drive biosynthesis. Individual members of the microbial world are extremely diverse and often unique with respect to their nutritional requirements and abilities. Hence, only the main patterns of the nutritional requirements and of behavior microorganisms will be delineated here.

Two approaches are traditionally taken to describe the nutritional behavior and requirements of living cells. The two approaches do not contrast, but rather complement each other. One is to categorize organisms on the basis of the principal sources of carbon and of energy they are able to use for growth; the other is to categorize them on the basis of quantitative and elemental aspects of the nutrients used for growth.

Nutritional Categories of Organisms

Based on their principal carbon and energy sources, microorganisms are classified into four different nutritional categories (**Table 1**). Most microorganisms using light as their principal source of energy are photoautotrophs, that is, they use an inorganic reduced compound as an electron donor and CO_2 as a carbon source (sometimes also referred to as photolithoautotrophs), whereas photoheterotrophs are a small group of specialists (certain purple and green bacteria). The ability to grow chemoautotrophically, that is, in the dark in a medium containing only inorganic nutrients, including a reduced inorganic compound as a source of energy, is specific for bacteria and archaea but is lacking in eukaryotic microorganisms. In these three types of nutrition the source for carbon and that for energy are clearly separated. This clear-cut distinction between the carbon and the energy source is not valid for the big group of chemoheterotrophic organisms that obtain their energy from the oxidation of reduced organic compounds and at the same time use them as a source of building blocks. The terms 'litho-' and 'organo-' are sometimes also used to indicate the source of hydrogen and electrons.

Some microbial strains are nutritionally rather flexible and could be placed into different nutritional categories. For example, the nutritional versatility of some photoautotrophic microalgae is such that they can employ a chemoheterotrophic lifestyle, growing in the dark at the expense of organic carbon sources, equally well. Also it should be mentioned that, when given the chance, most autotrophs can take up and assimilate considerable amounts of reduced organic compounds (not only growth factors, as described below) and use them to feed their anabolism. The nutritional category of such microorganisms is usually based on the simplest nutritional requirements, in which phototrophy and autotrophy precede chemotrophy and heterotrophy, respectively. The degree of nutritional flexibility, in addition, is indicated by describing strains as either obligate or facultative photo(chemo)autotrophs. In any of the four nutritional categories there are auxotrophic strains that require low amounts of specific organic compounds,

Table 1 Nutritional types of organisms based on the sources of carbon and energy used for growth

	Energy source	*Electron source*	*Carbon source*	*Nutrition type*
Phototrophs	Light	Reduced inorganic compound	CO_2	Photoautotroph (photolithoautotroph)
	Light	Reduced inorganic compound	Reduced organic compound	Photoheterotroph (photolithoheterotroph)
Chemotrophs	Chemical energy	Reduced inorganic compound	CO_2	(Chemolithoautotroph)
	Chemical energy	Reduced organic compound	Reduced organic compound	Chemoheterotroph (chemoorganoheterotroph)

that is, the growth factors. Unlike prototrophic strains, auxotrophs are unable to synthesize these growth factors from the principal source of carbon supplied in the medium.

Classes of Nutrients

In everyday usage the term 'nutrient' is restricted to compounds either fully or at least partly incorporated into cell material. However, biosynthesis requires energy in addition to building blocks. Frequently, the compounds involved in the generation of energy are not incorporated into biomass, but only take part in redox processes. Hence, based on their physiological purpose, compounds essential for microbial growth can be divided into two major groups (**Figure 1**):

1. Compounds that are either fully or partly incorporated into components of the biomass (nutrients), and
2. compounds that are not incorporated into biomass but are essential for the generation of energy (electron donors or acceptors).

This distinction cannot always be made in such a clear way because there are nutritional categories of organisms where some compounds can fulfill both functions at the same time. For example, reduced carbon sources are employed by chemoheterotrophs to obtain carbon precursors for biosynthesis as well as to generate energy, or, ammonia can be used by particular chemolithotrophs as a source of both energy and nitrogen.

The chemical elements contained in the nutrients consumed and incorporated into new cell material can be divided into five different classes. The division is mainly based on the quantities of these elements required for growth and their occurrence in dry biomass (**Table 2**). Not considered in this table is water, which is a main constituent of all cells, making up approximately 75% of the fresh cell weight. **Table 3** indicates that typically some 95% of the dry biomass is made up of the eight elements C, N, O, H, P, S, K, and Mg (class 1). These elements are indispensable for microbial growth. Class 2 elements are required in significant amounts, whereas those in class 3 and 4 are usually referred to as trace elements. For elements categorized in class 2 and 3 it can be demonstrated experimentally that they are essential, whereas it is difficult to prove that elements of class 4 are essential for growth. These elements are required in such low amounts that they are usually introduced into media with the bulk salts, where they are present as impurities or leach from the cultivation vessel and materials in contact with the medium. Finally, a special class of nutrients are the growth factors required by auxotrophic strains. This includes a diverse group of organic compounds. The physiological role of the main nutrients will be discussed in more detail in the section titled 'Requirements and physiological functions of principal elements'.

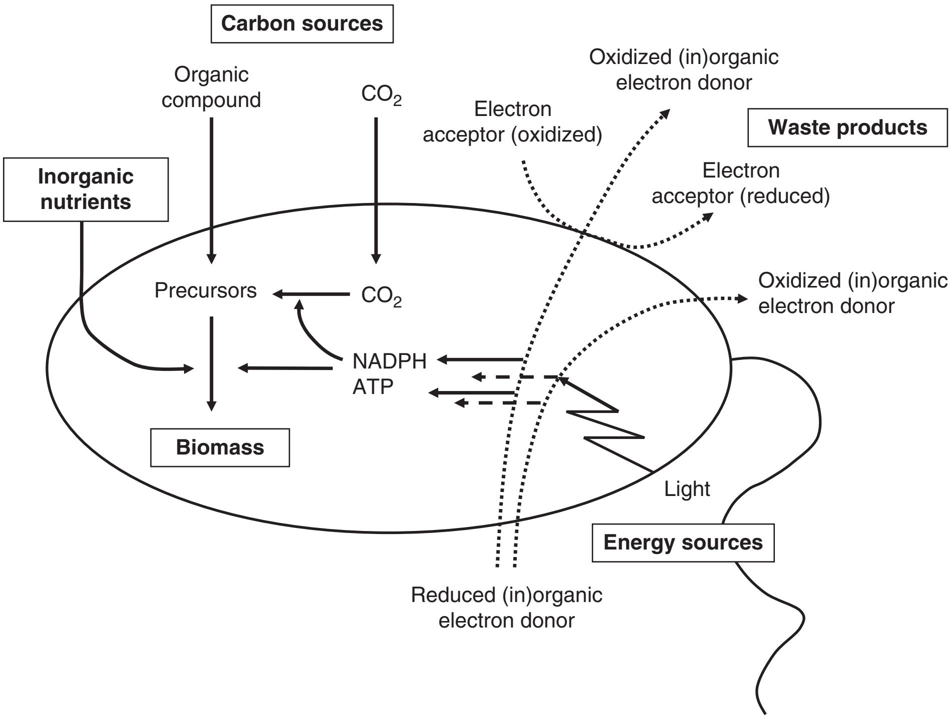
Carbon sources
Organic compound
CO_2
Oxidized (in)organic electron donor
Electron acceptor (oxidized)
Waste products
Inorganic nutrients
Electron acceptor (reduced)
Precursors
CO_2
Oxidized (in)organic electron donor
NADPH
ATP
Biomass
Light
Energy sources
Reduced (in)organic electron donor

Figure 1 Simplified sketch of the physiological function of nutrients for the growth of microorganisms.

Table 2 Classes of nutrients used for microbial growth based on their incorporation and occurrence in dry cell mass

Class 1	Always essential	Major elements: C, H, O, N Minor elements: P, S, K, Mg
Class 2	Mostly essential	Fe, Ca, Mn, Co, Cu, Mo, Zn
Class 3	In special cases essential	B, Na, Al, Si, Cl, V, Cr, Ni, As, Se, Sn, I
Class 4	Very rarely essential (difficult to prove)	Be, F, Sc, Ti, Ga, Ge, Br, Zr, W
Class 5	Growth factors, for special strains essential	Amino acids, purines and pyrimidines, vitamins, hormones, etc.

Based on Pirt (1975).

Table 3 Elemental composition of microbial biomass

	% of dry weight[a]		
Element in biomass	*Average*[b]	*Range*	*Typical sources utilized for growth in the environment*
Carbon	50	45[c]–58[d]	CO_2, organic compounds
Oxygen	21	18[e]–31[f]	H_2O, O_2, organic compounds
Nitrogen	12	5[d]–17[g]	NH_3, NO_3^-, organically bound N
Hydrogen	8	6[d]–8[g]	H_2O, organic compounds
Phosphorus	3	1.2[h]–10[i]	PO_4^{3-}, organically bound P
Sulfur	1	0.3–1.3	SO_4^{2-}, H_2S, organically bound S
Potassium	1	0.2[j]–5[k]	K^+ (can be replaced by Rb^+)
Magnesium	0.5	0.1[l]–1.1	Mg^{2+}
Calcium	1	0.02–2.0	Ca^{2+}
Chlorine	0.5		Cl^-
Iron	0.5	0.01–0.5	Fe^{3+}, Fe^{2+}, organic iron complexes
Sodium	1		Na^+
Other elements Mo, Ni, Co, Mn, Zn, W, Se, etc.	0.5		Taken up as inorganic ions

[a] Cells consist, on average, to 70% of their weight of water and 30% of dry matter.
[b] Gram-negative cells growing with excess of all nutrients at $\mu_{\max}$ in batch culture.
[c] Carbon-limited cells containing no reserve materials.
[d] Nitrogen-limited cells storing PHA or glycogen in the presence of excess C source.
[e] Cells grown N-limited accumulating neutral lipids.
[f] Cells grown N-limited accumulating glycogen.
[g] Cells growing at high μ containing high levels of rRNA.
[h] Cells grown P-limited.
[i] Cells accumulating the reserve material polyphosphate.
[j] Gram-positive *Bacillus* spores.
[k] Gram-positive bacilli.
[l] Magnesium-limited cells at low growth rates.
Data collected from Tempest (1969), Pirt (1975), Herbert (1976), and from results obtained in our own laboratory.

Elemental Composition of Biomass

The composition of a microbial cell is highly dependent on the cultivation conditions. The type of cellular constituents present (e.g., ribosomes, particular enzymes, membrane and cell wall components, compounds in the metabolic pool) and their amount can vary enormously. D. Herbert (1961) has emphasized this point saying that it is useless to give the cellular composition of a microbial cell without specifying both the exact growth conditions under which this cell has been cultivated and its growth history at the same time.

Despite this diversity and variability with respect to cell constituents, the elemental composition of microbial biomass – including cell material from archaea, eubacteria, and eukaryotes – varies in a surprisingly narrow range. This is documented in the overview of the average elemental composition of microbial biomass and its variability in **Table 3**. The relative constant composition of microbial biomass with respect to the major elements results from the fact that most of the dry biomass (typically some 95%) is made up of a limited number of organic macromolecules and only a small fraction can be attributed to monomers (metabolites and inorganic ions). Because protein, RNA, and phospholipids are the dominating components, massive changes in the content of a particular cell component are required before the overall elemental composition of the biomass is significantly affected (**Table 4**). For example, a significant increase in the carbon content of dry biomass is observed only when cells store high amounts of poly(3-hydroxyalkanates) (PHAs) or neutral lipids, whereas it is primarily the cellular oxygen content that is affected when

Table 4 Major polymeric constituents found in microbial cells and their average elemental composition

	% of dry weight							
Constituent	*Average*[a]	*Range*	*%C*	*%H*	*%O*	*%N*	*%S*	*%P*
Protein	55	15[b]–75	53	7	23	16	1	
RNA[c]	21	5[b]–30[d]	36	4	34	17		10
DNA[c]	3	1[b]–5[e]	36	4	34	17		10
Peptidoglycan	3	0[f]–20[g]	47	6	40	7		
Phospholipids	9	0[h]–15	67	7	19	2		5
Lipopolysaccharides	3	0[g]–4[i]	55	10	30	2		3
Neutral lipids		0–45[j]	77	12	11			
Teichoic acid[c,g]		0[i]–5[g]	28	5	52			15
Glycogen	3	0–50[j]	45	6	49			
PHB		0–80[j]	56	7	37			
PHA (C8)[k]		0–60[j]	68	9	23			
Polyphosphate[l]		0–20[m]			61			39
Cyanophycin[n]		0–10	42	15	25	27		

[a] Average composition of an exponentially growing Gram-negative cell (*Escherichia coli*) (Neidhardt, *et al*. 1990).
[b] Cells storing carbonaceous reserve materials.
[c] Inclusion of the highly negatively charged polymers such as RNA, DNA, polyphosphate, or cell wall components is paralleled by the presence of appropriate amounts of countercations, Mg^{2+}, Ca^{2+}, or K^{+}.
[d] At high growth rates.
[e] Cells growing slowly.
[f] Parasitic cell wall-less species.
[g] Gram-positive bacteria.
[h] Strains replacing phospholipids under P-limited growth conditions with P-free analogues.
[i] Gram-negative bacteria.
[j] Cells grown N-limited.
[k] PHA consisting of 3-hydroxyoctanoic acid.
[l] Grown P-limited.
[m] Some yeasts and bacteria.
[n] Some cyanobacteria contain the nitrogen storage material cyanophycin (asp-arg)$_n$.
Adapted from Herbert (1976) and extended. The figures given for the range have been collected from different organisms and, therefore, may not be applicable for particular strains.

cells accumulate glycogen. Note that an extensive incorporation of carbonaceous reserve materials results in a dilution and, hence, in a reduction of the relative content of other elements in dry biomass. A typical example is the reduced cellular nitrogen content found in cells accumulating PHA or glycogen.

Requirements and Physiological Functions of Principal Elements

Carbon

Dried microbial biomass consists of roughly 50% carbon, and virtually all of it is present as one of the many reduced organic cell constituents. Hence, as discussed (**Table 1**), the most obvious physiological function of carbon is as a source of building material for organic biomolecules. When its most oxidized form, CO_2, is used as the sole source of carbon for autotrophs, reduction to the level of organic cell material (usually at a redox state of carbon ~0) and the formation of carbon–carbon bonds is required. This process requires significant amounts of reducing equivalents (primarily NADPH) and energy (ATP) (see **Figure 1**). CO_2 is also employed as a terminal electron acceptor by methanogens and acetogens.

In contrast, heterotrophs use reduced carbon compounds to build their cell material and in most cases (an exception are the photoheterotrophs) the carbon compound fulfills a dual function, namely, it acts as both a carbon and an energy source. In some fermenting organisms reduced carbon compounds can act as terminal electron acceptors. Typically, heterotrophic cells utilize the same carbon source for both purposes, oxidizing a part of it to CO_2 (a process called dissimilation) and using the energy derived from this oxidation to synthesize cell material from the other part (assimilation). The ratio of dissimilated to assimilated carbon is essentially dependent on the degree of reduction of the carbon substrate used. The more oxidized the carbon compound, the more of it that has to be dissimilated in order to provide the necessary energy to drive the synthesis processes and the less of it that can be assimilated. This is reflected in the maximum growth yield observed for different carbon sources when plotted as a function of their energy content (i.e., their degree of reduction, or heat of combustion), as shown in **Figure 2**. Most extreme is the case of chemoheterotrophs growing at the expense of oxalate (HOOC–COOH). To generate energy, this compound is initially oxidized to CO_2, which is then assimilated in

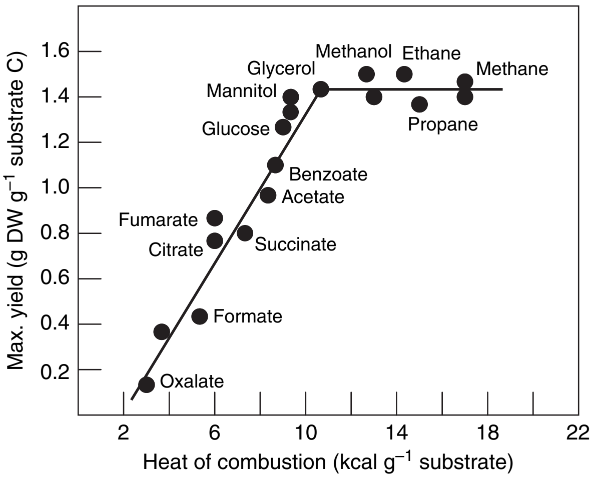

Figure 2 Maximum growth yields reported for various carbon substrates observed for heterotrophic organisms, as a function of the energy content of the carbon substrate. Adapted from Linton and Stephenson, 1978.

an autotrophic manner. The data depicted in **Figure 2** clearly demonstrate two different regimes of growth: first, a regime where growth is 'energy-limited – carbon excess' and the maximum yield increases linearly with the heat of combustion and, second, a growth regime of 'energy excess – carbon limitation' where the growth yield does not change anymore (in this case excess energy has to be dissipated as heat).

Heterotrophic microorganisms are extremely diverse with respect to the spectrum of carbon sources they can use for growth. Whereas some are restricted to only a few carbon compounds (e.g., some methanotrophs appear to utilize only methane and methanol), others are able to metabolize and assimilate more than a hundred different carbon compounds for growth. It should be added here that all heterotrophic organisms also assimilate a substantial amount of their cell carbon (typically 5–10%) from CO_2 (mainly for replenishing the tricarboxylic acid cycle when it is used as a source of building blocks for biosynthesis). Normally, this requirement for CO_2 is masked because CO_2 is produced in large amounts intracellularly from the catabolism of organic growth substrates. However, especially in freshly inoculated dilute cultures its absence can slow down, or even prevent, growth on organic substrates and some heterotrophic microorganisms even require elevated concentrations of CO_2 in the culture medium.

In case of energy excess and limited by an other essential nutrient, for example, nitrogen, carbon compounds can be stored intracellularly as reserve materials in the form of PHA, glycogen, or neutral lipids. In case of carbon starvation these internal carbon/energy sources are broken down to support cellular rearrangement and adaptation to the new conditions and to ensure survival.

Hydrogen

In cells, hydrogen is present in the form of water and as an element of all organic cell constituents. The main source of hydrogen for biosynthetic purposes is NADPH. The need for hydrogen is particularly evident for the reduction of CO_2 in autotrophs. In photo- and chemoautotrophs hydrogen equivalents used for CO_2 reduction can originate from water, from the oxidation of reduced inorganic compounds, or from reduced organic compounds. Chemoheterotrophs obtain their reducing equivalents from the oxidation of their primary carbon substrate.

Oxygen

As with carbon and hydrogen, oxygen is omnipresent in cells. It occurs in most of the organic components of cell material. The main sources of oxygen for the biosynthesis of particular cell components are water, molecular oxygen (but not in obligate anaerobes, where oxygen is frequently toxic) and, less obviously, CO_2. In aerobes, molecular oxygen is introduced into organic molecules with the help of mono- and dioxygenases. In addition to its function as a cell constituent, O_2 also serves as a terminal electron acceptor in aerobes.

Nitrogen

The cellular requirement for nitrogen is significant because it is a constituent of all major macromolecules (**Tables 3** and **4**). In cell components, nitrogen is mainly found in the reduced form (i.e., as primary, secondary, or tertiary amino groups). Oxidized forms (nitro and nitroso groups) are rarely found. Organic and inorganic forms of nitrogen at all states of oxidation, from NH_4^+ to N_2 to NO_3^-, can be used by microorganisms as sources of cell nitrogen (although some are unable to reduce oxidized forms). Note that the microbiological fixation of molecular nitrogen is of special interest to agriculture because of its ubiquitous availability in air. Frequently, microbial cells exhibit nitrogen requirements in the form of special amino acids (L-forms for incorporation into proteins, or D-forms for the synthesis of cell wall components) or peptides. Intracellularly, the assimilation of nitrogen occurs at the level of ammonia. Therefore, all higher-oxidized forms have to be reduced to this level before they can be used as a source of nitrogen.

Nitrogen compounds also play a major role in energy metabolism. Reduced forms (e.g., ammonia and nitrite) are used as sources of energy by nitrifying bacteria, whereas oxidized inorganic nitrogen compounds (e.g., nitrate, nitrite) are employed as terminal electron acceptors by denitrifying microbes.

Phosphorus

Inorganic phosphate is typically supplied in growth media as the only source of phosphorus. However, many organisms can also derive phosphorus from organic phosphates, such as glycerophosphate (note: organic P sources can be used to avoid precipitation of inorganic phosphate salts in a medium at basic pH values). Phosphate is primarily incorporated into nucleic acids, phospholipids, and cell wall constituents. Some organisms may also store it as polymetaphosphate, which can be reused as a source of internal phosphorus or for the generation of ATP. Intracellularly, the main fraction of phosphorus is contained in ribosomal RNA, whereas ATP and other nucleic acids make up only a minor fraction of the total cellular phosphorus.

Sulfur

The bulk of intracellular sulfur is found in proteins (cysteine and methionine). An important function of cysteine is its involvement in the folding of proteins by the formation of disulfide bridges. Frequently, these amino acids are also found in reactive centers of enzymes (e.g., in the coordination of reactive iron centers). The sulfur-containing coenzymes and vitamins (e.g., CoA, biotin, thiamine, glutathione, and lipoic acid) are small in quantity but physiologically very important. Intracellularly, sulfur is present in a reduced form (–SH) whereas it is usually supplied in growth media as sulfate salt. Some organisms are not able to catalyze this reduction and therefore must be supplied with a reduced form of sulfur, for instance with cysteine or H_2S.

Many inorganic sulfur compounds are also involved in the generation of energy. Whereas reduced sulfur compounds are used as electron donors (H_2S, thiosulfate, and S^0), oxidized forms are employed as terminal electron acceptors (SO_4^{2-}, S^0).

Major Cations

Magnesium

Magnesium is one of the major cations in cell material. Its intracellular concentration is proportional to that of RNA, which suggests that it is partly counterbalancing the negative charges of the phosphate groups in nucleic acids. Hence, its cellular concentration and requirement increase with growth rate. It is required for stabilizing the structure of ribosomes. Many enzymes are activated by or are even dependent on the presence of Mg^{2+}; some important examples are enzymes catalyzing reactions dependent on ATP or chlorophylls. Magnesium is also found bound to the cell wall and the membrane, where it seems to be responsible for stabilizing the structure together with other cations.

Interestingly, in Gram-negative bacteria the molecular ratio of Mg:K:RNA-nucleotide:PO_4 is always approximately 1:4:5:8 and is independent of growth rate, temperature, or growth-limiting nutrient. In Gram-positive organisms, this ratio is 1:13:5:13, except under phosphate-limited growth conditions in the chemostat, where it is 1:4:5:8. The higher K and PO_4 content of Gram-positive bacteria is due to the presence of phosphate-containing cell wall polymers (teichoic acids), which are replaced under phosphate-limited growth by non-phosphate-containing analogues (teichuronic acids).

Potassium

Potassium makes up a large part of the inorganic cations in biomass (**Table 3**). Only a small fraction of K^+ present in cells seems to be associated with binding sites of high affinity and specificity as it can be rapidly exchanged with other monovalent cations. A large fraction of K^+ is bound to RNA where it seems to have a stabilizing function. Therefore, as in the case of magnesium, its requirement increases with specific growth rate. Significant amounts are also found associated with the cell wall. K^+ activates a number of different enzymes, either nonspecifically (contributing to the ionic strength) or specifically (e.g., peptidyltransferase). Cations of similar size such as Rb^+ or NH_4^+ can frequently take over the function of K^+ (in contrast to magnesium, which cannot be replaced by other cations). Specific growth rates of many organisms are reduced when they are cultivated in media that are low in potassium. A variety of growth conditions affect the intracellular concentration of potassium, including osmolarity of the medium, temperature, pH, or sodium concentration. Therefore, this cation should always be added to growth media in significant excess.

Iron

Frequently considered a trace element, iron is used in significant amounts by virtually all organisms, not only by obligate aerobes (lactobacilli seem to be the only bacteria that do not need iron for growth). Iron is the catalytic center of a number of enzymes, especially those involved in redox reactions. Most essential are the various iron-containing cytochromes in the respiratory chain, flavoproteins, or the enzymes essential for the detoxification of reactive oxygen species such as catalase or superoxide dismutase. Many of the mono- and dioxygenases initiating the breakdown of pollutants are also iron enzymes. Most bacteria require concentrations of free iron exceeding 10^{-8} $\mathrm{mol\,l^{-1}}$ for growth. Iron(III), which is the species that prevails in aerobic environments, easily forms insoluble hydroxides and other complexes. Therefore, the acquisition of iron is a major problem for growing organisms. Many organisms react to iron limitation by excreting iron-complexing organic compounds with a high affinity toward iron, the siderophores. In mineral media, iron is therefore frequently supplied complexed with an organic ligand.

A number of anaerobic bacteria (in particular nitrate-reducing strains) can use Fe^{3+} (or Mn^{4+}) as a terminal electron acceptor, reducing it to Fe^{2+} (or Mn^{2+}). On the contrary, some specialist bacteria can use Fe^{2+} (or sometimes also Mn^{2+}) as a source of energy by oxidizing it to Fe^{3+}.

Calcium

In most organisms Ca^{2+} is present intracellularly in significantly lower amounts than Mg^{2+}, which has similar properties. The role of Ca^{2+} is not always clear; however, it seems to have important functions in stabilizing the cell wall and controlling membrane permeability. Changes in cell morphology and cell surface properties have been reported for several microorganisms in the absence of Ca^{2+}. Ca^{2+} activates many exoenzymes such as amylase. Furthermore, a number of uptake processes are stimulated by the presence of Ca^{2+}; an example is the uptake of exogenous DNA. It appears that extracellularly calcium plays the role that magnesium plays in the cytoplasm. Often Mg^{2+} cannot replace Ca^{2+} in these extracellular functions, but strontium can. In growth media attributing a clear function to calcium is difficult because of the presence of competing divalent cations that are essential for growth, such as Mg^{2+} or Mn^{2+}.

Trace Elements

Sodium

Microorganisms isolated from freshwater do not usually require sodium. For such organisms it is difficult to demonstrate that this cation is essential for growth because its requirements are low and sodium is present in all bulk salts as an impurity. However, some extremely halophilic microorganisms require high concentrations of NaCl for growth. For example, in order to not disintegrate, *Halobacterium* needs more than $2.5\,\mathrm{mol\,l^{-1}}$ NaCl in the growth medium. Sodium is also essential for certain photosynthetic bacteria and cannot be substituted for by other monovalent cations. In some marine bacteria, energy generation is even linked to the utilization of a Na^+ gradient rather than an H^+ gradient. Furthermore, in many microorganisms this ion is involved in the regulation of intracellular pH using a Na^+–H^+ antiport system.

Manganese

As a substitute for the iron-containing catalase, lactobacilli produce a manganese-containing pseudocatalase for protection against molecular oxygen. A high requirement for manganese is typical for lactic acid bacteria. Many of the lignolytic peroxidases contain manganese.

Cobalt

Co^{2+}-containing coenzymes and cofactors are widespread, the best known being the coenzyme B_{12}. Cobalamines (cobalt-containing biomacrocylic compounds) are found in bacteria as well as in humans, but the highest levels are usually found in methanogenic bacteria.

Nickel

Methanogens require unusually high amounts of Ni^{2+} for growth. It was found that it is a component of the coenzyme F430 in these organisms. Furthermore, this divalent cation is a constituent of virtually all hydrogenases in both aerobic and anaerobic microorganisms that either use or produce molecular hydrogen.

Copper

Copper is the key metal in the active center of many redox-reaction-catalyzing enzymes. It is present in many terminal oxidases of the respiratory chain. A number of other enzymes, such as peptidases, laccases, some nitrite reductases, or methane monooxygenase, contain Cu^{2+}.

Molybdenum

A whole family of enzymes, the molybdoenzymes (also referred to as molybdenum hydroxylases), contain Mo^{2+}. This family includes the central enzyme in the reduction of nitrate to nitrite: nitrate reductase. Furthermore, the nitrogen-fixing nitrogenase contains a MoFe cofactor, which is clearly different from the molybdenum cofactor shared by other Mo-containing enzymes.

Zinc

Many of the bacterial (extracellular) metalloproteases contain Zn^{2+} (e.g., elastase). Many of these proteases are produced by pathogenic strains and play an important role in the pathogenesis. Other zinc-containing enzymes are alkaline phosphatase and the long-chain alcohol dehydrogenases.

Feast and Famine: Unrestricted versus Nutrient-Limited Growth

In a typical laboratory shake flask culture all the nutrients supplied in a well-designed growth medium are initially present in excess and the cells grow exponentially at the highest rate possible under these conditions. However, in every environmental and technical system microbial growth cannot proceed unrestricted for a long time. A simple calculation makes this obvious: After 2 days of exponential growth a single microbial cell doubling every 20 min (as, e.g., *Escherichia coli* does) will have produced roughly 2×10^{43} cells. Assuming an average cell weight of

10^{-12} g this amounts to 2×10^{31} g of biomass, or approximately 4000 times the weight of the Earth. Hence, in every environmental and technical compartment, growth is always soon limited by the exhaustion of one or several nutrients.

The Concept of the Limiting Nutrient

The term 'limiting nutrient' is used with meanings, which, unfortunately, are frequently mixed up. The availability of nutrients can restrict the growth of microbial cultures in two distinct ways, namely stoichiometrically and kinetically. The stoichiometric limitation is defined by the maximum amount of biomass that can be produced from the limiting nutrient in this system ('Liebig's principle', from Justus von Liebig's agricultural fertilization studies around 1840, in which he found that the amount of a particular nutrient determined the crop on a field as long as all other nutrients were present in excess; eqn [1]). The kinetic limitation arises at low nutrient concentrations (typically in the low milligram to microgram per liter range) at which the (stoichiometrically) limiting nutrient also controls the specific rate of growth of cells (μ). This kinetic control of the specific growth rate usually follows saturation kinetics and the Monod equation (eqn [2]) is typically used to describe the relationship between concentration of the growth rate-controlling nutrient (usually referred to as substrate S) and μ.

$$x = x_0 + (s_0 - s) \cdot Y_{X/S} \qquad (1)$$

$$\mu = \mu_{\max} \frac{s}{K_s + s} \qquad (2)$$

where s_0 is the initial and s the actual concentration of the limiting nutrient S; x is the dry biomass concentration and x_0 the initial dry biomass concentration; $Y_{X/S}$ is the (dry biomass) growth yield for nutrient S; μ is the specific growth rate and $\mu_{\max}$ the maximum specific growth rate; and K_s is the apparent Monod substrate affinity constant.

This is visualized in **Figure 3** for growth in a closed batch culture system in which the cells initially grow unrestricted until the consumption of the limiting nutrient first leads to growth at a reduced rate, and then to growth stoppage. The limiting nutrient determines the final concentration of biomass that can be reached. In flow-through systems, such as a continuous culture, in which fresh medium is continuously added and surplus culture removed, the rate of addition of limiting nutrient (thought to be a single compound) simultaneously controls μ and the concentration of biomass obtained in the culture (see 'Continuous Cultures (Chemostats)'.

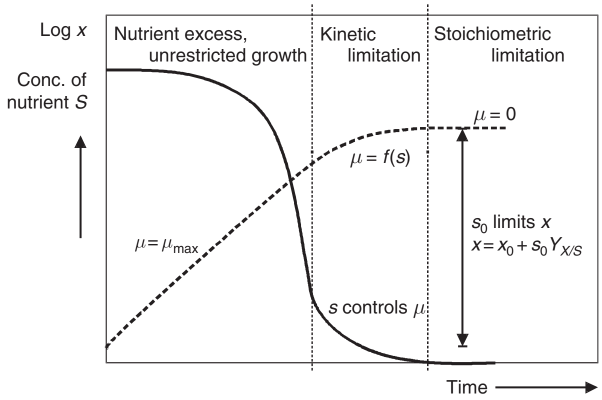

Figure 3 Kinetic and stoichiometric limitation of microbial growth in a batch culture by the concentration of the limiting nutrient (substrate) S. s_0, initial concentration of S; s, actual concentration of S; x, biomass concentration; x_0, initial biomass concentration; $Y_{X/S}$, growth yield for nutrient S.

In laboratory cultures, it is possible to cultivate cells under well-defined conditions where the growth-limiting nutrient is known when employing defined synthetic media. Quantitative and practical aspects of nutrients in microbial growth media for controlled cultivation will be discussed in the section titled 'Design and analysis of defined minimal growth media'. For the cultivation of heterotrophic microorganisms for research purposes and the production of biomass, media with a limiting carbon and energy source, with all other nutrients being supplied in excess, are commonly designed. However, in biotechnological processes, limitation by nutrients other than carbon is frequently employed to manipulate the physiological state and metabolic performance of microbial cultures. Restriction (limitation) of specific nutrients induces or enhances formation of many microbial metabolites and enzymes. Examples are the increased productivity in antibiotics fermentation by growth in phosphate-limited media, the production of citric acid under Fe-, Mn-, and/or Zn-limited batch culture conditions, the synthesis of NAD under Zn–Mn limitation, and the accumulation of the intracellular reserve materials polyhydroxybutyrate (PHB) or PHA (bioplastic) by limiting the supply of nitrogen.

In contrast to cultivation in the laboratory and industrial scale, it is difficult to assess the nutritional regimes that govern growth of microbial cells in environmental systems (especially the identification of the kinetic control). In aquatic systems, primary microbial production, that is, autotrophic growth, is usually limited by the availability of phosphorus, whereas the growth of heterotrophs is limited by the availability of a complex mixture of carbon energy sources. There are indications that microbial growth is frequently controlled not by a single nutrient, but by combinations of two or more nutrients simultaneously; in addition to phosphorus and carbon energy sources, nitrogen and iron may frequently be colimiting.

Growth Limitation and Growth Patterns during Batch Cultivation

Mostly, microbiologists cultivate their strains in batch culture, that is, closed systems such as shake flasks or agar plates, where after inoculation no additional nutrients are added (except for oxygen in aerobic cultures). Usually, the carbon energy source is selected as the limiting nutrient when cultivating heterotrophs. In defined mineral media with a single growth-limiting carbon energy source, this results in the typical pattern found in all textbooks, with an exponential phase, in which cells grow at a constant specific growth rate, and a distinct and quick switch from exponential growth to the stationary phase, as illustrated in **Figure 4**.

However, batch growth patterns depend very much on the nature of the growth-limiting nutrient. An example is given in **Figure 5** for the growth of *Klebsiella pneumoniae* in synthetic medium with glucose as the sole carbon energy source where either glucose (**Figure 5(a)**) or phosphate (**Figure 5(b)**) is limiting growth. During exponential growth, with all nutrients in excess, the specific growth rate μ is identical in both cultures. In the case of glucose limitation, the consumption of sugar to completion brings biomass production to an immediate standstill and a distinct switch from the exponential to the stationary phase; the kinetic limitation phase as indicated in **Figure 3** is hardly visible. Acetate produced during the exponential phase is now consumed but does not lead to a detectable further increase in biomass concentration. The growth pattern in a phosphate-limited batch culture is distinctly different in as much as not only growth but also glucose consumption and acetate production proceed after phosphate is consumed to completion. In this example, biomass increased almost threefold, and was paralleled by acetate excretion, until limited availability of glucose slowed down growth. In this phase of obvious extracellular phosphate limitation the cell redistributes intracellularly bound phosphorus from nonessential cell components to places where it is absolutely required, such as DNA. This phase of growth is characterized by high intracellular dynamics of cell composition and extensive cell rebuilding. Hence, limitation by nutrients different from carbon does not usually result in a complete stoppage of growth. Whereas for elements that are covalently linked like C, N, S, and P, a break in the growth curve indicates limitation and a change in metabolism, this is usually not visible for nutrients that are not covalently linked in cell components (such as Mg^{2+}, K^{+}) or may be redistributed easily (such as iron); here, the onset of limitation is hardly detectable from the biomass growth curve and hidden limitations of growth can be overlooked easily.

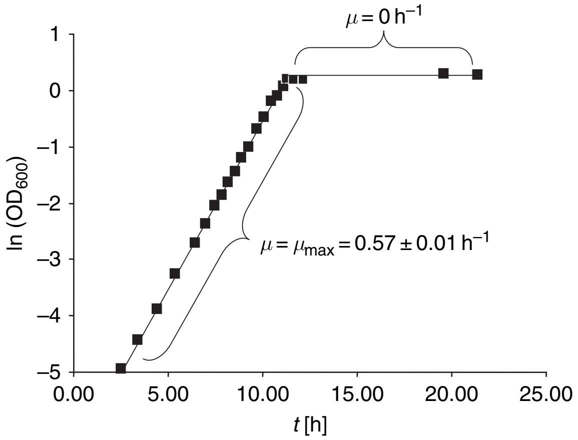

Figure 4 Growth of *Escherichia coli* K-12 MG1655 in batch culture with glucose mineral medium (37 °C) in a shake flask. The initial glucose concentration was 1.25 g l^{-1}; growth was measured spectrophotometrically as optical density at 600 nm (OD_{600}) and the specific growth rate (μ, h^{-1}) was calculated from 3–5 adjacent data points. Unpublished data from J. Ihssen, M. Berney, and T. Egli.

Often, standard complex media, for example, Lysogeny Broth (LB, also known as Luria-Bertani medium), are widely used in molecular microbiology to cultivate microbial strains in the laboratory (**Table 5**). Such media consist of chemical or enzymatic digests of plant, yeast, or animal tissues and contain a plethora of organic compounds utilizable by many microorganisms for growth. Nevertheless, most of the organic molecules of different size and quality present in such media cannot be accessed for growth; for example, *E. coli* is able to use only roughly 10% of the total carbon supplied in LB with 90% remaining unutilized. In such complex mixtures, easily accessible carbon compounds are utilized first, supporting fast growth, and – as growth proceeds – cells switch to less preferred, more complex compounds that support lower specific growth rates. This is illustrated in **Figure 6** for the growth of *E. coli* in LB where the specific growth rate starts out at 2.5 h^{-1} (which is a doubling time of less than 17 min) and then continuously decreases until stationary phase is reached. Hence, the true exponential growth phase with a constant μ is limited to the very first part of the curve. Thus, one has to be cautious when interpreting data obtained under such growth conditions because many of the cellular parameters investigated are μ-dependent. Growth under natural conditions is probably very similar to growth in complex media; also, in nature a mixture of carbonaceous compounds supports growth of heterotrophic microorganisms and growth is limited by available carbon energy sources most of the time. This is shown in **Figure 7** for the growth of *E. coli* with pasteurized natural freshwater assimilable organic carbon (AOC) from a small river. Because of methodological restrictions, information on the growth of microbes on the pool of natural carbon energy sources is still severely limited. However, recent experiments done in our laboratory demonstrate that growth at environmental carbon concentrations (100–200 μg AOC l^{-1}) is still extremely efficient and

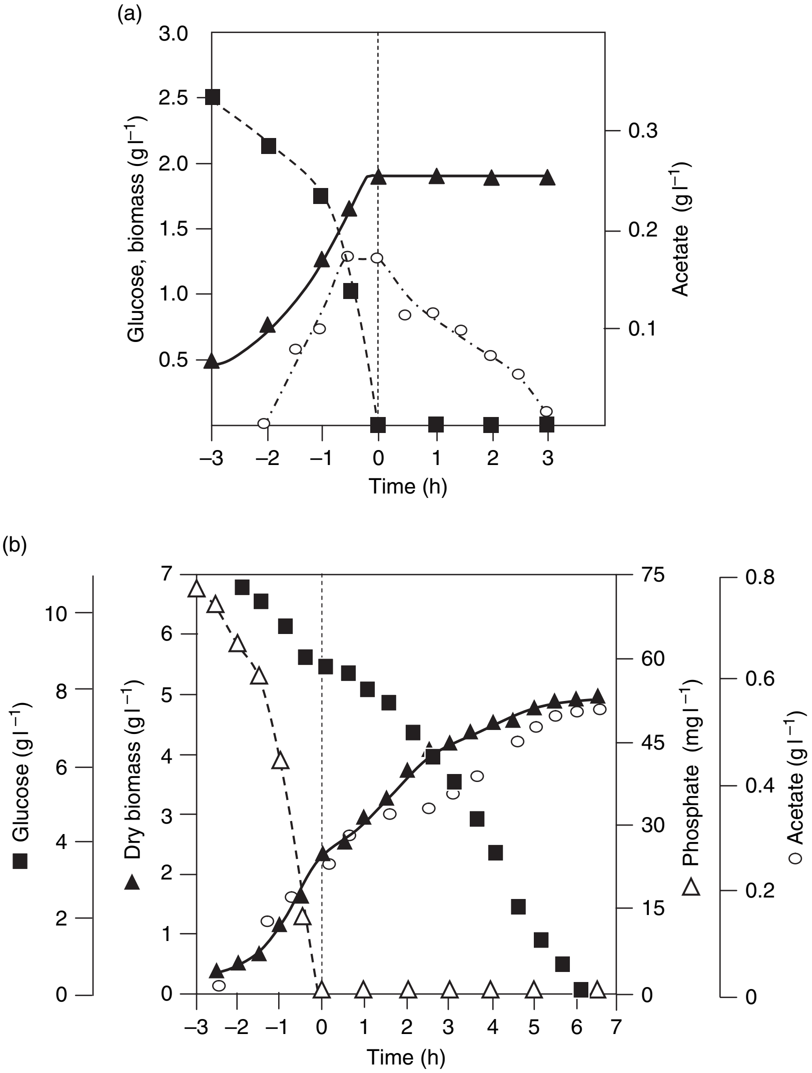

Figure 5 Growth, glucose consumption, and acetate production in a batch culture of *Klebsiella pneumoniae* cultivated in a synthetic glucose medium where either (a) glucose or (b) phosphate is the growth-limiting nutrient (30 °C, pH 7.0). Growth is given as dry biomass produced. Adapted from Wanner U and Egli T (1990) Dynamics of microbial growth and cell composition in batch culture. *FEMS Microbiology Reviews* 75: 19–44.

Table 5 Composition of a selection of media used to set the maximum specific growth rate of *Salmonella typhimurium* in batch culture

No	*Medium*	*Comments*[a]	μ_{max} (*h*$^{-1}$)
1	Brain + heart infusion	Full strength	1.94
5	Nutrient broth	Diluted 1:2 with medium No 14	1.80
6	Nutrient broth	Diluted 1:5 with medium No 14	1.66
7	Casamino acids	1.5% + 0.01% Tryptophan in medium No 14	1.39
9	20 Amino acids	20 Natural amino acids + mineral salt solution[a]	1.27
10	8 Amino acids	8 Natural amino acids + mineral salts solution[a]	1.01
14	Glucose salt	0.2% Glucose + mineral salts solution[a]	0.83
15	Succinate salt	0.2% Succinate + mineral salts solution[a]	0.66
19	Methionine salt	0.06% Methionine + mineral salts solution[a]	0.56
22	Lysine salt	0.014% Lysine + mineral salts solution[a]	0.43

[a] Mineral salts solution contained $MgSO_4$, Na_2HPO_4, $Na(NH_4)HPO_4$, KCl and citric acid as chelating agent. It did support no visible growth without addition of a carbon source.

Reproduced from Schaechter M, Maaløe O, and Kjeldgaard NO (1958) Dependency on medium and temperature of cell size and chemical composition during balanced growth of *Salmonella typhimurium*. *Journal of General Microbiology* 19: 592–606.

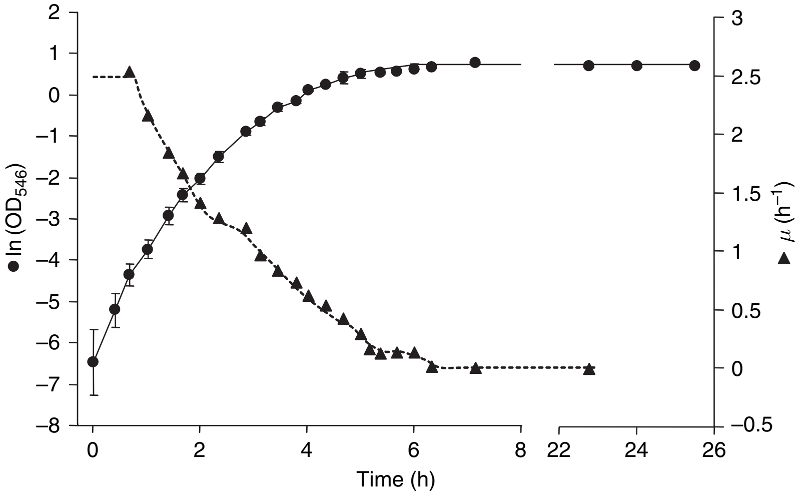

Figure 6 Growth of *Escherichia coli* K-12 MG1655 in batch culture with complex medium (LB, 37 °C) in a shake flask. Growth was measured spectrophotometrically as optical density at 546 nm (OD$_{546}$) and the specific growth rate (μ, h^{-1}) was calculated from 3–5 adjacent data points. Values are means (± standard deviations, error bars) from three experiments. Adapted from Berney, *et al*. (2006) *Applied and Environmental Microbiology* 72: 2586–2593.

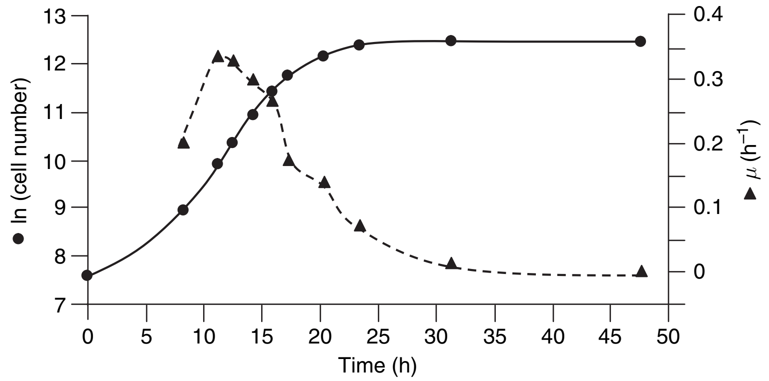

Figure 7 Growth of *Escherichia coli* O157 in a batch culture with assimilable organic carbon from pasteurized freshwater. Growth at 30 °C was measured flow cytometrically as the increase in cell concentration after staining cells with SYBR Green and the specific growth rate (μ, h^{-1}) was calculated from 2–3 adjacent data points. Unpublished data from M. Vital, F. Hammes, and T. Egli.

heterotrophic freshwater communities are able to form on average 10^7 cells from 1 μg of AOC. Because of the much bigger cell size of *E. coli*, this yield is reduced and ranges within 1–2 × 10^6 cells per 1 μg of AOC.

Design and Analysis of Defined Minimal Growth Media

To grow and synthesize their own cell material, organisms must obtain all the required building blocks (or their precursors) and the necessary energy from their environment. Consequently, to cultivate microbial cells in the laboratory these nutrients must be supplied in a culture medium in adequate amounts and in a form accessible to the organism. Investigations of stoichiometric aspects of microbial growth can be traced back to the first formulation of defined growth media by Pasteur in the 1850s and Raulin, a former student of Pasteur, reported in 1869 the first cell yield coefficients for C, N, P, K, Mg, Fe, and Zn for an *Aspergillus* species.

As a result of the physiological diversity of the microbial world, a myriad of media of different compositions have been published, for either selective enrichment or cultivation of particular microorganisms. All these media contain components the nutritional function of which is obvious, in particular when considering their elemental or energetic function. Nevertheless, most nutritional studies undertaken have been qualitative rather than quantitative and different nutrients have been added in more or less arbitrary amounts. Also, many of the media contain components and the reason for their inclusion cannot be clearly identified because their inclusion is based more on experience or tradition than on a clear purpose.

The identification of nutritional requirements of microbial cells usually calls for the use of defined synthetic media. The design of defined culture media is based on quantitative aspects of cell composition and it allows influencing the growth of a microbial culture at three major levels:

- First, the choice is made as to which nutrient is to limit the growth of the culture stoichiometrically and kinetically.
- Second, for nutritionally flexible microbial strains, the choice is made as to which type of metabolism the organism should resort to by the selection of the compounds that are supplied to fulfill a particular nutritional requirement, including electron donors and acceptors.
- Third, and often linked with the second point, the choice of the maximum specific growth rate to be achieved during unrestricted growth in batch culture is set.

Setting $\mu_{\max}$ During Unrestricted Growth

In addition to physico-chemical parameters such as temperature or pH, the maximum specific growth rate of a microorganism is influenced by the composition of the nutrients supplied in the medium. This has been elegantly illustrated for the growth of *Salmonella typhimurium* by Schaechter and colleagues (1958), who used 22 media of different compositions to obtain growth of the culture at differing rates under nutrient excess conditions (a selection is given in **Table 5**). Although the four media supporting the highest specific growth rates are undefined, the other media consist of a minimal salt medium to which different carbon sources or amino acid mixtures are added. Hence, selection of the quality of precursors supplied in the mineral medium allowed adjustment of the specific growth rate of the culture in a defined and reproducible way. For metabolically flexible microbes this choice can be extended to the level of electron acceptors or donors.

Medium Design and Experimental Verification of the Limiting Nutrient

Designing a growth medium

In the design of a defined growth medium the initial decisions to be made are the choice of the maximum concentration of biomass the medium should allow to produce $x_{\max}$, and the selection of the nature of the growth-limiting nutrient (according to Liebig's principle). Typically, defined growth media for heterotrophic microbes are designed with a single carbon energy source restricting the amount of biomass that can be produced, whereas all other nutrients (each of them usually added in the form of a single compound) are supplied in excess. Having set $x_{\max}$, it is possible to calculate the minimum concentration of the different elements necessary in the culture medium to produce $x_{\max}$, using the individual average elemental growth yields ($Y_{X/E}$). To ensure an excess of all the non-limiting nutrients in the medium, their concentrations are multiplied by an excess factor (F_E). In this way, the concentrations of the different nutrients required in the growth medium (e_{req}) are present in a theoretically x-fold excess with respect to the limiting nutrient:

$$e_{\text{req}} = \frac{x_{\max}}{Y_{X/E}} \cdot F_E \qquad (3)$$

An example of the design of a carbon-limited medium supporting the production of $10\,\mathrm{g\,l^{-1}}$ of dry biomass of is given in **Table 6**. Note that in this medium the ingredients are chosen in such a way that it is possible to change the concentration of each of the elemental nutrients individually (e.g., by including $MgCl_2$ plus $NaHSO_4$ instead

Table 6 Design of a carbon-limited minimal medium allowing the production of $10\,\mathrm{g\,l^{-1}}$ of dry biomass

Medium constituent	*Source of, function*	*Growth yield assumed (g dry bio-mass/g element)*	*Excess factor assumed with respect to carbon*	*Mass of element (g l^{-1})*	*Mass of constituent (g l^{-1})*
Glucose	C, energy	1	1	10	25.0
NH_4Cl	N	8	3	3.75	14.33
NaH_2PO_4	P	33	5	1.52	5.88
KCl	K	100	5	0.5	0.95
$NaHSO_4$	S	100	5	0.5	1.87
$MgCl_2$	Mg	200	5	0.25	0.98
$CaCl_2$	Ca	100	10	1.0	2.77
$FeCl_2$	Fe	200	10	0.5	1.13
$MnCl_2$	Mn	10^4	20	0.02	0.046
$ZnCl_2$	Zn	10^4	20	0.02	0.042
$CuCl_2$	Cu	10^5	20	0.002	0.0042
$CoCl_2$	Co	10^5	20	0.002	0.0044

Based on elemental growth yields obtained from the composition of dry biomass (see **Table 1**).

Based on Pirt (1975) and Egli and Fiechter (1981). Elemental growth yields for C and the trace elements Zn, Cu, Mo, and Mn were taken from Pirt (1975). Excess factors for different elements were chosen taking into account their variation observed in dry biomass.

of $MgSO_4$). In addition, this medium is only weakly buffered; hence, it might be necessary to control the pH during growth.

This approach works well for the design of media for the cultivation of aerobic microorganisms at low to medium biomass concentrations. More problematic is the design of media for anaerobic cultures and cultures that require an alkaline pH for growth, where many of the medium components precipitate easily at the required redox potential or pH. Similar problems arise for high cell density cultures where solubility or toxicity problems of some of the medium ingredients have to be taken into account (see 'Some practical comments on the preparation of media').

An estimate for most of the elemental growth yield factors $Y_{X/E}$ can be obtained from an elemental analysis of dry biomass cultivated under unrestricted growth conditions in batch culture (compare **Table 3**). For carbon, oxygen, and hydrogen $Y_{X/E}$ cannot be calculated directly from the elemental composition of cells because these elements are not only incorporated into the biomass, but also serve other metabolic functions. For example, carbon is not only assimilated by heterotrophs but also oxidized to CO_2 to supply energy (see also **Figure 2**). Also, not included in this table is the amount of electron acceptor that has to be supplied to ensure growth. **Table 7** shows the yield coefficients for oxygen, for some of the other common electron acceptors, and for some electron donors that support chemolithotrophic growth.

Two points influence the choice of excess factors. First, for elements whose cellular content does not vary considerably as a function of cultivation conditions, excess factors can be set low (N, P, and S), whereas for elements that are known to vary considerably (e.g., with growth rate), they are set higher. Second, the chemical behavior of the medium component in the growth medium also has to be taken into account for choosing F_E. For example, most of the trace elements easily precipitate in growth media at neutral and basic pH and as a result their biological availability is reduced (and difficult to assess). Therefore, they are added in a 10- to 20-fold excess despite the fact that a metal complexing agent is usually added to the medium to keep them in solution.

For biotechnological purposes, for which batch and fed-batch processes are primarily used, it would be advantageous to design media that contain all the nutrients in exactly the amount required, so that all nutrients would be consumed to completion at the end of the process. This, however, is difficult to achieve owing to the variability of the yield factors for the individual elements, their dependence on the cultivation conditions, and the necessity to always ensure availability. Nevertheless, one of the most important points in medium optimization in biotechnology is to optimize the consumption of nutrients and minimize their loss.

Some practical comments on the preparation of media

It is appropriate to add a few comments on some of the most important precautions to be taken when preparing a growth medium. Many sugars easily deteriorate during sterilization at basic pH (especially in the presence of phosphates and peptones). This leads to a browning of the medium. The products formed can be inhibitory for growth. This can be avoided by sterilizing the medium at a slightly acidic pH, or by sterilizing the sugars separately from the medium.

It is well known that all trace metals easily form highly insoluble phosphate salts and precipitate in growth media. This can be avoided by the addition of metal-chelating agents such as ethylene diamine tetra-acetic acid (EDTA), Nitrilotriacetic acid (NTA), or alternatively also carboxylic acids such as citrate or tartrate. The addition of chelating agents has a twofold effect: On the one hand, it prevents the precipitation of trace metals; on the other hand, it acts as a sink for these metals, and in this way reduces their toxicity by lowering their free (for the microbes accessible) concentration.

At medium pH >7, the alkaline earth metals calcium and magnesium (as the trace metals) easily precipitate in

Table 7 Some growth yield factors for electron donors and electron acceptors

Electron donors	Molecular hydrogen	$Y_{X/H_2} \approx 12\,g\,mol^{-1}$
	Thiosulfate	$Y_{X/S_2O_3} \approx 4\,g\,mol^{-1}$
	Fe^{2+}	$Y_{X/Fe_2^+} \approx 0.35\,g\,mol^{-1}$
	NH_4^+ to NO_2^-	$Y_{X/NH_4^+} \approx 1.3–2.6\,g\,mol^{-1}$
	NO_2^- to NO_3^-	$Y_{X/NO_2^-} \approx 0.9–1.8\,g\,mol^{-1}$
Electron acceptors	Molecular oxygen	$Y_{X/O_2} \approx 10^a–42^b\,g\,mol^{-1}$
	NO_3^- to N_2	$Y_{X/NO_3} \approx 27\,g\,mol^{-1c}$
	NO_2^- to N_2	$Y_{X/NO_2} \approx 17\,g\,mol^{-1c}$
	N_2O to N_2	$Y_{X/N_2O} \approx 9\,g\,mol^{-1c}$
	SO_4^{2-} to H_2S	$Y_{X/SO_4} \approx 5–10\,g\,mol^{-1}$

[a] For growth with reduced substrates such as methane or n-alkanes.

[b] For growth with more oxidized substrates such as glucose.

[c] For growth of *Paracoccus denitrificans* with glutamate as carbon substrate.

the presence of phosphate (or in the presence of carbonate ions when using a bicarbonate-buffered medium, or if only hard water is available) to form highly insoluble phosphate salts. These precipitates are sometimes difficult to see with the eye, especially in shake flasks owing to the small volume of the medium. To avoid this, the medium can be sterilized at a slightly acidic pH (which requires the possibility that the pH can be adjusted later) or the phosphate salts can be sterilized separately from the rest of the medium and combined after cooling. For an elaborate treatment on this subject and more detailed information, especially on some of the established media, the reader is referred to the review by Bridson and Brecker (1970).

Experimental identification of growth-limiting nutrient

The variability of yield factors for the different nutrients, depending on the organism used and the compounds included in a medium, requires that the nature of the growth-limiting nutrient be experimentally verified for each case. For this, the maximum concentration of biomass (*x*) that can be produced in such a medium is determined as a function of the initial concentration of the medium component (s_0) that is supposedly growth-limiting, with the concentration of all other medium components kept constant. This experiment can be done in either batch or continuous culture.

The typical (theoretical) relationship obtained in such an experiment is visualized in **Figure 8**. Ideally, the relationship between s_0 of a growth-limiting nutrient and *x* is initially a straight line that passes through the origin, that is, when no growth-limiting nutrient is added to the medium no biomass is produced. When s_0 exceeds a certain concentration, a deviation from the linear relationship is observed. It is at this concentration that another nutrient becomes growth-limiting. Deviations from this relationship can be observed when one of the bulk salts used for medium preparation contains low amounts of the limiting nutrient as an impurity, or when a certain amount of the limiting nutrient becomes inaccessible in the medium (e.g., due to precipitation with another medium component). Note that variations in pH due to increasing concentrations of biomass or excreted toxic products can affect cultivation conditions and also influence biomass yield.

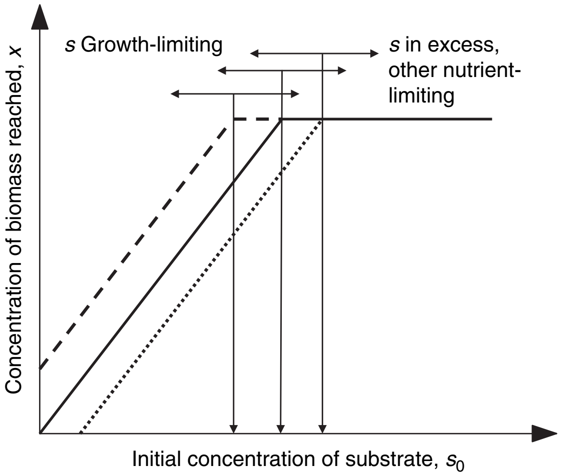

Figure 8 Concentration of dry biomass (*x*) that can be produced in a medium, as a function of the initial concentration of the growth-limiting substrate (s_0). (- - - - - -), *x* in a case where low amounts of the limiting substrate are introduced with an other medium component; (. . . . . . .), *x* in a case where part of the limiting substrate is not available for the cells, for instance, due to precipitation with another medium component.

In practice the linear relationship between *x* and s_0 is often observed, although interpretation of the data is frequently not as straightforward as suggested by **Figure 8**. This is demonstrated in **Figure 9** for *Pseudomonas oleovorans* growing in a continuous culture at a fixed dilution rate with a mineral medium in which either carbon or nitrogen was limiting growth, depending on the ratio of the two nutrients. By keeping the concentration of ammonia constant and increasing the concentration of the carbon source this bacterium was cultivated at different C:N feed ratios; the resulting biomass and the ammonia and carbon source concentrations were measured at the steady state. When *P. oleovorans* was cultivated with citric acid as the sole source of carbon energy the steady-state biomass concentration in the culture initially increased linearly with the concentration of the carbon source (**Figure 9(a)**). Accordingly, the residual concentration of excess nitrogen decreased in the culture broth with increasing C:N feed ratios. At a C:N feed ratio of ~8.5, ammonia was consumed to completion. A further increase of the carbon concentration in the feed medium led to no further increase of the biomass produced. Instead, excess citric acid accumulated in the culture. Thus, one growth regime clearly limited by carbon with nitrogen in excess, and one limited by nitrogen with carbon in excess, can be recognized. When the same experiment was performed with octanoic acid as the sole carbon source the biomass concentration also increased initially when growth was carbon-limited with excess nitrogen present in the culture (**Figure 9(b)**). At a C:N feed ratio of 7.0, the residual concentration of nitrogen in the culture became undetectable. Despite this, the concentration of the biomass in the culture continued to increase linearly when the concentration of octanoate was further increased in the feed medium, with all the carbon consumed to completion. Only when the C:N feed ratio in the feed exceeded 14.5 did unutilized octanoate become detectable in the culture liquid. Thus, based on the pattern of biomass concentration, growth became nitrogen-limited above a C:N feed ratio of 15.3, whereas the residual concentration of the nitrogen source in the

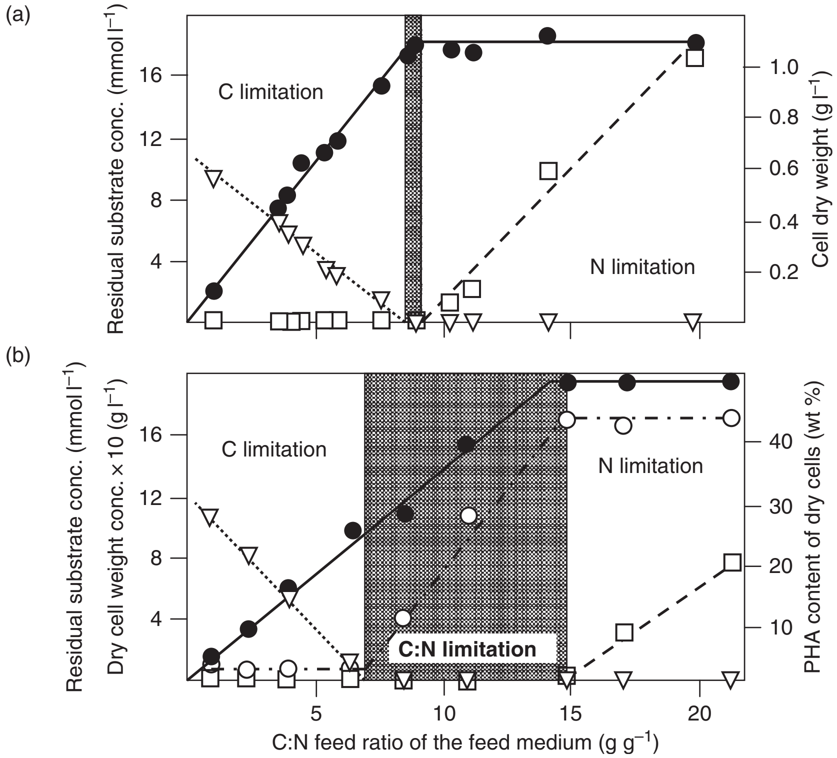

Figure 9 Growth of *Pseudomonas oleovorans* with either (a) citrate or (b) octanoate as the sole source of carbon in continuous culture at a fixed dilution rate of 0.20 h^{-1} as a function of the C:N feed ratio of the feed medium. The C:N feed ratio of the feed medium was varied by keeping the concentration of the nitrogen source (NH_4^+) constant and changing the concentration of the carbon source. Adapted from Durner R, *et al*. (2001) *Biotechnology and Bioengineering* 72: 278–288. ● indicates cell dry weight; ▽, ammonium-N concentration; □, concentration of (a) citric acid or (b) octanoic acid in the culture; ○, polyhydroxyalkanoate (PHA) content of cells.

culture indicated that the limitation of the culture by nitrogen had already occurred at C:N feed ratios higher than 7.0. The analysis of the cells showed that the effect observed was a result of channeling the surplus carbon into the formation of the reserve material polyhydroxyalkanoate (in other organisms this may be PHB glycogen, or lipids). This dual-nutrient-(carbon/nitrogen)-limited growth regime is always observed when the organism has the ability to store carbonaceous reserve materials. Here, the extension of this zone between the two single-nutrient-limited growth regimes depends on the storage capacity of the organism for reserve material and the growth rate. Such multiple-nutrient-limited zones are observed not only for the interaction of carbon with nitrogen but also for other combinations of nutrients, such as C–P, C–Mg, or N–P. Furthermore, the extension of this zone is determined by the limits a microorganism exhibits with respect to its elemental composition under differently limited growth conditions.

Thus, even when a linear relationship is obtained for the biomass, care has to be taken in the interpretation. In such a case, it is advantageous to know which of the nutrients is the second limiting component in the medium.

Assessing the Quality of Media and Some Notes of Caution

Above approach can be used not only to design growth media limited by a specifically selected nutrient, but also to assess the quality of various media. (Never assume that growth media reported in the literature are perfect. Many times they are not, nor are they necessarily employed for the purpose they were designed for.) Such an assessment usually provides a good understanding about the capacity of a growth medium with respect to the maximum biomass that it can support, the nature of the limiting nutrient, and the degree of excess of other nutrients.

Note that most of the classical media used before the 1960s did not include trace elements. Their addition was usually not necessary because they were contained as impurities in the bulk minerals used for the preparation of the medium. Modern media are frequently prepared with 'ultrapure' salts, and, not surprisingly, they fail to support good growth unless they are amended with a trace element solution. A typical example is the classic synthetic medium M9 that is used widely for growth of *E. coli* in genetic studies. This medium in its original composition does not support growth of *E. coli* for more than a few generations, after which growth slows down and finally comes to a halt.

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Gram-Negative Opportunistic Anaerobes: Friends and Foes

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Defining Statement
The Complex Relationship Between the Normal Human Microbiota and Human Disease
Human Colonic *Bacteroides* Species in Health and Disease
Koch's Postulates and Microbiota Shift Diseases (Dysbiosis)
Common Themes
Further Reading

Glossary

anaerobe Bacterium that is unable to divide in the presence of oxygen, usually having a fermentative metabolism.

biofilm A multilayered community of bacteria that is held together by a polysaccharide matrix; may contain more than one species of bacteria.

colonic fermentation Fermentation of dietary and host polysaccharides by colonic bacteria to produce short-chain fatty acids that are absorbed and utilized by colonic mucosal cells.

conjugation, conjugative transfer The process by which two bacterial cells make close contact with each other and transfer DNA from one to the other; segments of DNA can be quite large.

capsule A complex matrix composed of polysaccharide or protein that covers the surface of the bacterial cell.

dysbiosis Change in the composition of a microbiota that has adverse consequences for the host.

microbiota Complex bacterial population that is normally found in a site on the human body; usually protective or neutral with respect to human health.

microbiota shift disease (dysbiosis) Disease that results from a shift in the composition of the microbiota rather than from invasion by a single pathogen.

reservoir hypothesis Bacteria in the human colon can acquire or donate antibiotic resistance genes across species and genus lines to other colonic bacteria or bacteria passing through the area.

Abbreviations

ICE	integrated conjugative element
LPS	lipopolysaccharide
PCR	polymerase chain reaction

Defining Statement

Gram-negative anaerobic bacteria that normally colonize areas of the human body such as the mouth and colon can cause human disease in two ways: First, if these bacteria escape from the site they normally occupy, they can cause abscesses in virtually any organ of the body. Second, shifts in the composition of the microbiota of sites where the bacteria are normally found can have pathological consequences, a phenomenon called dysbiosis. These bacteria are not always harmful. For the most part, they are innocuous or play a positive role in human health.

The Complex Relationship Between the Normal Human Microbiota and Human Disease

The human body normally harbors complex bacterial populations in the mouth, colon, and vagina. All of these populations contain obligate anaerobes, and in the colon, the anaerobes are the dominant bacterial group. For the most part, the bacteria in these sites are beneficial, but under some conditions members of these populations can cause disease. Bacteria escape from these areas into sterile areas such as blood and tissue, where they can cause infections in almost all areas of the body. Most of the anaerobic bacteria that cause disease are Gram-negative. At one time, many physicians believed that obligate anaerobes could not cause infections in humans because they thought of the human body as an aerobic environment. In fact, the perception of the body as a completely aerobic environment is not entirely correct. For one thing, the concentration of free oxygen in blood is relatively low. For another, anaerobic pathogens can thrive in regions of damaged tissue. These regions are anoxic because the blood supply that would keep them oxygenated is cut off. This also protects the bacteria from the cells of the innate and adaptive immune systems because the cells and proteins that comprise these systems cannot reach the interior of the region. The result is the formation of abscesses that can occur in almost any organ of the body. Bacteria leaking from these abscesses into the bloodstream cause bacteremia and sepsis.

A disease state can also arise if the composition of the microbiota shifts substantially. Two well-documented examples of this are periodontal disease and bacterial vaginosis. In both of these conditions, the shift is from a predominantly Gram-positive to a predominantly Gram-negative microbiota. In periodontal disease, the Gram-negative bacteria are predominantly anaerobes. In the case of bacterial vaginosis, the Gram-negative bacteria are facultative bacteria that are beyond the scope of this article. Why and how such shifts occur is not clear. Antibiotic use is unquestionably one of the forces that can cause a microbiota shift to occur, but there must be other factors because microbiota shift diseases can occur in people who are not using antibiotics.

Oral Gram-Negative Anaerobes

Periodontal disease

Bacteria in the mouth form biofilms on tissue or teeth in order to avoid being washed out of the site by the flow of saliva or ingested liquids. Biofilms that form on teeth are called plaque. The plaque that occupies the exposed portion of a tooth is composed mainly of facultative Gram-positive lactic acid bacteria such as *Streptococcus* species. Even in this seemingly aerobic site, however, obligate anaerobes can be found, probably because the formation of a biofilm inhibits the influx of oxygen or oxygen is consumed by some organisms in the biofilm.

Above the gum line, where the environment is more anoxic, the plaque contains more obligate anaerobes. The development of periodontal disease is associated with a shift in the composition of plaque to one that is dominated by Gram-negative anaerobes.

The species of bacteria that are most commonly associated with periodontal disease are listed in **Table 1**. Complete genome sequences are available or under way for representatives of all of these species.

Table 1 Bacterial species most commonly associated with periodontal disease

Species	*Most closely related genus or phylogenetic group*
Porphyromonas gingivalis	Human colonic *Bacteroides* spp.
Prevotella intermedia and other *Prevotella* spp.	Human colonic *Bacteroides* spp.
Fusobacterium nucleatum	Gram-positive bacteria *(Firmicutes)*
Actinobacillus actinomycetemcomitans[a]	Pasteurellaceae, especially *Haemophilus* spp.

[a] Facultative anaerobe.

Porphyromonas gingivalis is the best studied of the species listed in **Table 1**. It is a rod-shaped bacterium that forms black-pigmented colonies. The pigmentation is due to its use of hemin rather than free iron as an iron source. *P. gingivalis* is a member of the phylum Bacteroidetes. Its closest relatives are *Prevotella* spp. and human colonic *Bacteroides* spp. This association was first inferred from 16S rRNA gene sequence analysis, but has been confirmed by comparisons of whole genome sequences.

P. gingivalis is most associated with chronic adult periodontitis, a condition that is characterized by inflammation of the gums. This species has also been proposed as a possible cause of bad breath. In fact, you can buy online a small can labeled ‘bad breath’ that contains what is supposed to be a stuffed representation of *P. gingivalis,* although it looks nothing like the actual bacterium.

P. gingivalis produces a number of virulence factors that may explain its role in periodontal disease. Fimbriae allow the bacteria to attach to the tooth surface and to oral tissue. Others trigger inflammation. The bacteria produce polysaccharidases and an impressive variety of proteases. The polysaccharidases and proteases degrade many of the components of the intracellular matrix. Tissue destruction caused by these enzymes may make a major contribution to the inflammation that is seen in periodontal disease patients. Some of the proteases attack complement components and other proteins of the host’s innate response system, possibly impairing the ability of the host to eliminate the bacteria. *P. gingivalis* also produces a polysaccharide capsule. This capsule stimulates an inflammatory response. Finally, *P. gingivalis* has been shown to invade tissue culture cells. Whether this trait also contributes to the virulence of the organism *in vivo* is still not clear.

Prevotella intermedia is associated with acute necrotizing gingivitis and periodontal disease with bone loss. Like *P. gingivalis*, *P. intermedia* forms black-pigmented colonies and produces a variety of proteases. Much less is known about this species than about *P. gingivalis*, but it is beginning to receive more attention. In culture, the two organisms form aggregates, an observation that is consistent with the formation of a multispecies biofilm on the teeth. The biofilm itself may help to protect the bacteria from the host's defense responses.

Fusobacterium nucleatum has an interesting phylogenetic placement. It stains Gram-negative and clearly has an outer membrane, but both its 16S rRNA gene sequence and its genome sequence place it in the Gram-positive bacteria. *F. nucleatum* also has an unusual role in periodontal disease. Most workers in the field do not consider it a pathogen in the sense that it causes inflammation. Rather, *F. nucleatum*'s role seems to be to aid in the formation of the biofilm by attaching to other bacteria such as *P. gingivalis*. Because of this propensity, it has been called a 'bridge organism'. Although *F. nucleatum* is not considered an oral pathogen, it is capable of causing abscesses in the head, neck, chest, lung, and abdomen if it escapes from the mouth.

Actinobacillus actinomycetemcomitans is included in **Table 1** for completeness, even though it is a facultative anaerobe rather than an obligate anaerobe. It is associated with localized aggressive periodontitis. In periodontal lesions, it is seen aggregating with *F. nucleatum*. A change in name from *Actinobacillus* to *Aggregatibacter* has been proposed. The closest phylogenetic relatives of *A. actinomycetemcomitans* are members of the Pasteurellaceae.

Other diseases

Anything that causes the gums to bleed, including vigorous toothbrushing, dental manipulations, or periodontal disease, will allow bacteria to enter the bloodstream. In most people, bacteria are rapidly eliminated and do not cause an infection. If the bacteria are not eliminated, they can cause infections in any organ of the body. As already mentioned, the oral Gram-negative anaerobes can cause abscesses in many parts of the body. Associations between oral Gram-negative anaerobes and other diseases have been suggested but are more controversial. The two diseases that have received the most attention are atherosclerosis and premature birth. *P. gingivalis* stimulated the accumulation of aortic plaque in a mouse model of atherosclerosis. A possible explanation of this result is that *P. gingivalis* can infect and inflame damaged tissue, where the plaque is forming in the aorta. Whether *P. gingivalis* is a significant contributor to atherosclerosis is uncertain. The current leading bacterial candidate for involvement in heart disease is *Chlamydia* spp. But since evidence of *P. gingivalis* has been found in human aortic plaque, it might play some role. Many people would like to believe that there is a bacterial component of atherosclerosis because if so the disease might be treatable with antibiotics.

F. nucleatum causes premature birth in mice. In this animal model, the bacteria lodge in the placenta, where they elicit an inflammatory response that is presumably the cause of premature birth or direct damage to the fetus. *F. nucleatum* has also been isolated from human amniotic fluid and placental tissue of women who experienced premature birth. So far, however, making a convincing epidemiological connection between periodontal disease and premature birth or other complications of pregnancy has proven difficult. This could be due to the fact that even if periodontal disease is a cause of premature birth, it is only one of many causes.

Colonic Gram-Negative Anaerobes

In the colon, Gram-negative anaerobes comprise one of the two numerically predominant bacterial groups, accounting for 20–30% of colonic bacteria found in that site. Most of these Gram-negative bacteria are members of *Bacteroides* species. The other major group of colonic bacteria comprises a number of species of Gram-positive anaerobes.

Normally, *Bacteroides* species have a beneficial effect on colonic health because they compete with and suppress the growth of pathogens such as *Clostridium difficile*. Also, they are major players in the colonic fermentation, a process in which polysaccharides from the diet or from the body itself are fermented to produce short-chain fatty acids. Colonic cells absorb these fatty acids and use them as sources of carbon and energy. If the colon is ruptured, however, *Bacteroides* can escape into normally sterile areas of the body such as blood and tissue, where they can cause life-threatening infections. The Gram-positive anaerobes, by contrast, rarely cause extraintestinal infections.

In earlier times, the release of bacteria from the colon resulted in a rapidly developing condition called peritonitis. This condition is caused by *E. coli* and related bacteria that are present in low numbers in the colon. When antibiotics became available, physicians found that by administering antibiotics immediately after such breaches as a ruptured appendix or a suspected surgical perforation of the colon, *Escherichia coli* became a much less common cause of this type of sepsis. *Bacteroides* spp., however, proved to be resistant to the antibiotics that controlled *E. coli*, and a much more slowly developing form of sepsis was seen. Not only could *Bacteroides* spp. cause bloodstream infections but they could also cause abscesses in almost any organ of the body.

More recently, attention has been turned to shifts in the composition of the microbiota of the colon that alter the balance between *Bacteroides* species and the Gram-positive anaerobes. Such shifts are now suspected of contributing to a variety of human diseases ranging from inflammatory bowel disease to obesity, although such connections are still controversial.

Human Colonic *Bacteroides* Species in Health and Disease

Bacteroides spp. were the first Gram-negative anaerobes to be taken seriously as human pathogens. This historical placement, taken together with a long-term fascination with the contents of the human colon as a factor in human health as well as disease, has produced more detailed information about *Bacteroides* spp. than is available for the other species of Gram-negative anaerobes. Accordingly, it is worth considering this genus and its relationship with the human body in some detail.

The Definition of *Bacteroides* as a Genus and a Phylogenetic Group

Bacteroides species were the first Gram-negative anaerobes to be viewed as potentially serious pathogens. Accoridngly, more is known about their traits and ability to cause disease than about the other Gram-negative anaerobes. In the premolecular era, the definition of *Bacteroides* as a genus was problematic. *Bacteroides* were defined are Gram-negative obligate anaerobes that were nonmotile and did not produce spores. *Bacteroides* spp. used carbohydrates as energy sources and produced acetate, propionate, and succinate. This was a rather vague definition. Even the end products that defined *Bacteroides* spp. were not very informative in the sense that they were produced by many anaerobic bacteria. Many of the oral anaerobes such as *Porphyromonas* spp. and *Prevotella* species were originally classified as *Bacteroides* spp. Differences between the oral species and the colonic *Bacteroides* spp. had already been noted. Most evident was the black pigmentation of the oral strains. Also, they were much less aerotolerant. A careful phylogenetic analysis based on 16S rRNA genes has finally sorted out these different genera.

A result of the early phylogenetic analysis that was surprising at the time was the realization that *Bacteroides* and related genera were members of a completely separated phylogenetic group from the more familiar enterics and other proteobacteria. For a long time, the big phylogenetic divide was thought to be the divide between the Gram-negative and Gram-positive bacteria. After all, these two groups had very different cell wall structures. To find that within the Gram-negative bacteria, there were even bigger phylogenetic divides was unexpected. Now, of course, it is well known that there are numerous phyla of Gram-negative bacteria. The Gram-positive bacteria are unusual in that they seem to cluster in a single phylum.

A number of genome sequences of members of the Bacteroidetes are beginning to appear. These genome sequences confirm the original 16S rRNA conclusions about the relatedness of the various genera in this group. A notable feature of the genomes of such numerically major species as *Bacteroides thetaiotaomicron* is the presence of numerous glycosidases and polysaccharidases, a finding consistent with the well-known ability of many *Bacteroides* species to use oligo- and polysaccharides as sources of carbon and energy.

The Normal Life of *Bacteroides* spp. in the Human Colon

Gram-negative anaerobes are found in far fewer numbers than Gram-positive bacteria in the oral cavity, whereas *Bacteroides* spp. account for about 20–30% of the colonic microbiota. The Gram-positive bacteria are still the numerically predominant group but they do not outnumber the Gram-negative anaerobes by the huge margin seen in the mouth. One might consider the numerical predominance of *Bacteroides* species in the colon to be a virulence factor of sorts. If it were not for their success in colonizing the human colon, these species would have far fewer opportunities to cause infection. Perhaps the ability of *Bacteroides* to colonize the human colon in such high numbers should be more accurately called an 'opportunity factor'. As we are beginning to learn in the modern era of bacterial pathogenesis studies, opportunity factors may be at least as important, if not more so, than have been called virulence factors.

Colonization of the colon is not, however, the only factor responsible for their ability to cause infection because other equally numerous *Bacteroides* species, such as *Bacteroides vulgatus* and *Parabacteroides distasonis*, cause human infections much less commonly than the much less numerous *Bacteroides fragilis*, which is considered the main cause of *Bacteroides* infections.

Normally *Bacteroides* and the other numerically predominant groups of colonic bacteria are beneficial. *Bacteroides* spp. make a major contribution to colonic fermentation. *Bacteroides* spp. are saccharolytic, but simple sugars such as glucose and maltose are not available in the colon because they are absorbed during passage through the small intestine. Two types of carbohydrates reach the colon: plant cell wall polysaccharides (also known as dietary fiber) and host-derived substances such as mucins and mucopolysaccharides. Mucin is a complex mixture of polysaccharides and proteins that lubricate the intestinal tract and form a protective barrier that helps prevent bacteria in the colon lumen from attaching to the colonic mucosa and thus having more of a chance to invade colonic cells and pass through the colon wall. Mucopolysaccharides are also polysaccharide–protein complexes but they have a different purpose. Mucopolysaccharides are part of the extracellular matrix that attaches human cells to each other in the tissue. So when turnover of the small and large intestinal mucosal cells sloughs large numbers of these cells into the intestinal lumen, mucopolysaccharides are also released.

Products of the colonic fermentation, mainly acetate, propionate, and butyrate, are absorbed across the colonic mucosa and act as sources of carbon and energy for colonic cells and underlying tissue. The contribution of the colonic fermentation to the human carbon and energy budget has been estimated to be about 8–10%, although this estimate is more of a guess than a concrete measurement. The human body must constantly produce new colonic mucosal cells and slough them into the colon lumen to prevent bacteria from adhering and invading. Thus the products of the colonic fermentation may strike an energy balance with the human body in which colonic bacteria 'pay rent' for being allowed to remain in the location.

If you are intrigued by the hypothesis that a shift in the colonic microbiota contributes to obesity, the colonic fermentation could become an important consideration. The obesity connection is alleged to result from a shift in the ratio of Bacteroidetes to Firmicutes (Gram-positive bacteria) to favor the Firmicutes. In theory, an increase in the efficiency of the colonic fermentation, even if it is only a few percent, could lead over time to increases in weight. Since so little is known about the Gram-positive anaerobes that comprise the majority of the Firmicutes, it is premature to speculate on their contribution to colonic fermentation.

A confusing aspect of the obesity hypothesis is that, traditionally, a diet high in meat and fat "Western diet", not a high-fiber diet, has been associated with obesity. Yet, the high-fiber diet should contribute the most to an increase in the colonic fermentation because of the high content of dietary polysaccharides. Evidently, a connection between obesity and the ratio of Bacteroidetes to Firmicutes may be more complex than the current simple hypothesis. One factor that may prove important is that a 'Western' diet contains many fabricated foods that have a high concentration of polysaccharides such as guar gum, which maintain the texture of puddings and ice cream. These are much more readily fermented than the less soluble polysaccharides such as cellulose.

It is probably the case that virtually all of the *Bacteroides* in the colon are located in the lumen of the colon, not attached to the colonic wall. Colonic bacteria account for about one-third of the volume of colonic contents. This concentration of bacteria is so high that there is no room for all of them to lodge in the mucin layer or to attach to the colon wall. Many *Bacteroides* cells appear to be attached to plant particles or are free-living in the colon interior. This does not preclude the possibility that a few *Bacteroides* species preferentially colonize the mucin layer or even reach the mucosal surface.

A considerable amount of information is available about the mechanism of polysaccharide utilization by *Bacteroides* spp. *Bacteroides* appear to specialize in the utilization of soluble polysaccharides and are generally unable to metabolize such insoluble substrates as cellulose. The colon is a highly competitive environment, so it is not surprising that *Bacteroides* spp. that utilize polysaccharides do not excrete polysaccharide-degrading enzymes into the extracellular environment. Instead, they bind the polysaccharide to the bacterial outer membrane and somehow transport it into the periplasmic space where the degradative enzymes are located. In this way, the products of the degradative enzymes are sequestered by the bacterium that made them.

Virulence Factors of Human Colonic *Bacteroides* spp.

Normally, virulence factors are defined as toxic molecules such as protein toxins or traits of the organism such as capsules that protect them from the defenses of the human body. Virulence features of the human colonic *Bacteroides* spp. are more ill-defined and their role seems to be to elicit an inflammatory response. Also, they need to survive to locate the sites of damaged tissue where they can thrive. Although *Bacteroides* species cannot grow in the presence of oxygen, many of them are able to survive low levels of oxygen for prolonged periods. Pathogenic *Bacteroides* species such as *B. fragilis* have a complex response to oxygen that is probably protective, and this may also be true of other *Bacteroides* species. The ability to survive in the bloodstream, where low levels of oxygen may be encountered, would allow the bacteria to find and lodge in small areas of damaged tissue where conditions do support growth.

It is possible that the ability to degrade polysaccharides such as mucopolysaccharides and other human cellular polysaccharides, a trait that contributes to their ability to colonize the colon, may act as virulence factors. These enzymes could help to break down tissues at the margins of an abscess, thus allowing the bacteria to expand the area of damage.

Clearly, however, the main virulence factor of *B. fragilis*, the most common cause of *Bacteroides* infections, is a capsule, that consists of two polysaccharides: PSA and PSB. Capsules of better-studied pathogens have as their function preventing phagocytic cells such as neutrophils from engulfing and killing the bacteria. This does not seem to be the main role of the *Bacteroides* capsule. This capsule, which consists of two charged polysaccharides, seems to have an inflammatory activity that makes a major contribution to abscess formation. Paradoxically, this capsule or at least PSA has been shown to have a protective role in a mouse model of inflammatory bowel disease. In this case, PSA from bacteria in the intestinal lumen affect inflammatory cells in the intestinal lining in such a way as to restore the normal balance of immune function.

Bacteroides species, like other Gram-negative bacteria, have lipopolysaccharide (LPS) molecules that are located in their outer membranes and are able to elicit an inflammatory response. Compared to *E. coli* LPS, *Bacteroides* LPS is much less toxic when injected into mice.

The most commonly cited *Bacteroides* infections are abscesses, but some strains of *B. fragilis* are able to cause diarrheal disease in the intestine because they produce a small protein enterotoxin. Recently, this enterotoxin, which has a protease activity, has been shown to act by cleaving the zonal occludens, proteins that bind intestinal cells together to form an impermeable barrier. This finding raises the possibility that *Bacteroides* strains might produce other, as-yet-undiscovered, protein toxins. So far, genes that are recognizable as toxin genes have not been found in the available *Bacteroides* genome sequences.

Antibiotic Resistance Genes and the Reservoir Hypothesis

There is still disagreement about whether to regard antibiotic resistance genes as virulence genes, but there is no question that one of the reasons *Bacteroides* species have been such successful opportunists is their resistance to antibiotics. Some resistance are based on failure to take up an antibiotic. Perhaps the most obvious of these is the universal resistance of *Bacteroides* spp. to aminoglycosides such as streptomycin and gentamicin. Aminoglycosides inhibit protein synthesis, but to do so they have to enter the cell. Apparently, the resistance of *Bacteroides* spp. to aminoglycosides is due to the failure to take up the antibiotic.

Two antibiotics that were identified early as being particularly effective against *Bacteroides* spp. were clindamycin and metronidazole. Clindamycin is a lincosamide that inhibits protein synthesis and metronidazole has a nitro group that is reduced inside the cell to a form that damages DNA. Both of these antibiotics are more active against anaerobes than facultative bacteria. Clindamycin resistance is due to the acquisition of a gene that encodes a methyltransferase that modifies rRNA, the part of the ribosome that binds clindamycin. This prevents clindamycin from binding to the ribosome and stopping protein synthesis. *Bacteroides* strains have also become resistant to more familiar antibiotics such as penicillins and cephalosporins, macrolides such as erythromycin, and tetracyclines. These resistances are also due to the acquisition of resistance genes. The tetracycline resistance genes do not encode efflux proteins such as those commonly found in the enterics, but rather encode proteins that interact with the ribosome to prevent tetracycline from binding. Resistance to all of these antibiotics has been increasing. Resistance to tetracycline is now so widespread in *Bacteroides* spp. that tetracycline is no longer considered to be a drug of choice for treating *Bacteroides* infections. An interesting but little known fact is that *E. coli*, which is susceptible to most aminoglycosides when growing under aerobic conditions becomes much more resistant to them when grown under anaerobic conditions.

Bacteroides species can mutate to resistance, but more commonly they become resistant by acquiring resistance genes via horizontal gene transfer, which occurs primarily by conjugation. Not only does horizontal gene transfer occur within a matter of hours but more than one resistance gene can be acquired at the same time. Even if a resistance gene is not expressed initially in a new host, selection pressure can easily produce mutations in the promoter that render the gene active.

For many years, conjugative gene transfer was considered to be mediated entirely by plasmids. Only when attention shifted to *Bacteroides* spp. and to Gram-positive bacteria did it become obvious that there are also integrated gene transfer elements. These were originally called conjugative transposons, which is something of a misnomer because these elements are more like lysogenic bacteriophage than like transposons. More recently, a new nomenclature has been suggested: integrated conjugative elements or ICEs. *Bacteroides* also have conjugative plasmids, but these seem to be making less of a contribution to horizontal gene transfer among members of this group than ICEs.

Conjugative transposons are normally integrated into the bacterial chromosome. To transfer, they excise to form the circular transfer intermediate. A single-stranded copy of this transfer intermediate is transferred to the bacterial recipient, then the circular forms reintegrate into the chromosome.

Recent studies that suggest that conjugative transfer occurs frequently in the human colon have led to the suggestion of another pathogenic aspect of *Bacteroides* spp., the ability to serve as a reservoir for antibiotic resistance gene. This idea has been called the reservoir hypothesis and is illustrated in **Figure 1**. Conjugative transfer of DNA can cross species and genus lines. Numerous reports of the same resistance genes in both Gram-positive and Gram-negative bacteria as well as in different species of the same genus supports the hypothesis that such broad host range events occur in nature and occur fairly frequently. The reservoir hypothesis states that the dominant members of the colonic population, Gram-positive anaerobes as well as *Bacteroides* species, collect transmissible resistance genes and spread them to other bacteria.

This includes not only bacteria that are normally resident in the colon but also bacteria such as some of the Gram-positive pathogens that are swallowed or ingested in food and pass through the colon. The normal residence

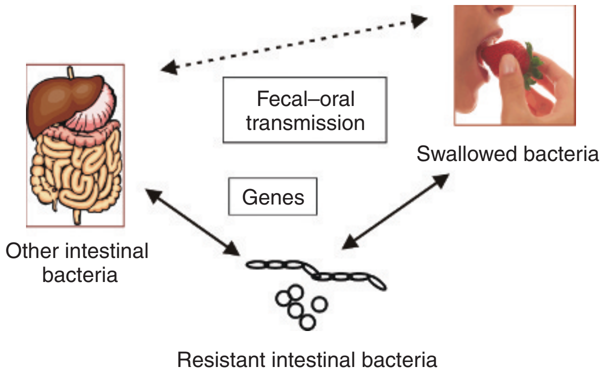

Figure 1 Reservoir hypothesis. Bacteria in the colonic microbiota can contribute to the ability of other bacteria to cause disease by transferring genes to other colonic bacteria or to swallowed bacteria that are merely passing through the colon. Transient bacteria are excreted but can be spread to other areas of the body through the fecal–oral route or directly to skin. These bacteria can then go on to cause disease in another body site. This hypothesis is best established for antibiotic resistance genes but could involve genes that increase the ability of the bacteria to cause disease (virulence genes).

time of a bacterium passing through the colon (24–48 h) is more than sufficient for conjugative transfer of resistance genes to occur. Thus, a swallowed bacterium such as *Staphylococcus aureus* or *Streptococcus pneumoniae* might acquire resistance genes during passage through the colon. After excretion, these bacteria enter the environment and could then recolonize human body sites from which they could later cause infections if the body's defenses were impaired.

Koch's Postulates and Microbiota Shift Diseases (Dysbiosis)

Throughout this article, a unique feature of the Gram-negative anaerobes has been mentioned repeatedly: their participation in shifts in the composition of the microbiota that can lead to a disease state. This phenomenon represents a paradigm shift away from the traditional view of infectious diseases as caused by single organisms. In fact, associations between shifts in the microbial populations of the colon or the mouth and human disease have been difficult for some scientists to accept because it is so difficult to establish a cause and effect relationship.

How are we to cope with such a complex picture of disease? During the 1800s, scientists interested in bacterial diseases focused on diseases caused by single bacteria. A microbiologist of that period, Koch, developed a set of guidelines for establishing a cause and effect relationship between a bacterium and a disease. This set of guidelines is now called Koch's postulates. The first postulate was that the bacterium had to be associated with the lesions of the disease. The second was that the bacterium must be isolated from infected blood or tissue in pure culture. The third, which has proven problematic even in some cases of single-microbe infections, is that the isolated bacterium must be shown to cause the disease if inoculated into a human or animal model. The fourth is that the bacterium must be reisolated from the infected human or animal in pure culture.

How would one approach developing a set of Koch's postulates for microbiota shift diseases? Currently, such postulates are formulated as follows: First, the composition of the bacterial population associated with the disease must be established. Since it is often the case that many of the bacteria in a site have not been cultivated, this is normally done using DNA-based (culture-independent) methods such as polymerase chain reaction (PCR) amplification and sequencing of 16s RNA genes. This in effect combines Koch's first and second postulates. The third postulate could be satisfied by inoculating germfree rodents, rodents raised in sterile conditions so that they have no normal bacterial microbiota, to give them an aberrant or normal bacterial population.

Engineering microbiota diversity in mice is the strategy used in recent studies linking a shift in the colonic microbiota to obesity. Results of these studies provide support for the cause and effect relationship between a shift in the colonic microbiota to one in which *Bacteroides* spp. decline in abundance and the concentration of the Gram-positive anaerobes rises. This pattern is the opposite of the shift to a primarily Gram-negative population that has been associated with periodontal disease.

A similar strategy of using gnotobiotic mice as a model for dysbiosis is also seen in studies that attempt to link a shift in the microbiota of the colon to inflammatory bowel disease. In this case, however, the interaction may be more complex. Although early studies emphasized the negative effects of the Gram-negative anaerobes in inflammatory bowel disease, a very recent study claims that the *B. fragilis* capsule, normally thought of as a virulence factor, may have a protective effect in inflammatory disease. A problem with these murine studies, of course, is that inflammatory disease in mice may not be as good a mimic of the human disease as scientists hope.

In humans, one might use antibiotic intervention or some other treatment such as probiotics to restore the microbiota to normal and at the same time to reduce the symptoms of the disease. Unfortunately little is known about how to manipulate the human microbiota in this way to achieve the desired population, and so ethical concerns could arise.

Common Themes

Both the oral and colonic Gram-negative anaerobes illustrate that bacterial pathogens can act in more complex than were envisioned by early microbiologists and even by scientists who founded the era of modern bacterial pathogenesis. In this paradigm, bacterial pathogens usually came into a susceptible person from the environment or from other people. These pathogens produced fairly distinct diseases, and thus had readily identifiable virulence factors. This view of disease was first challenged by the so-called opportunists, bacteria such as *S. aureus*, *Staphylococcus epidermidis*, *S. pneumoniae*, and *Pseudomonas aeruginosa* that were normally harmless but were able to take advantage of breaches in the defenses of the human body.

The Gram-negative anaerobes have pushed the envelope of opportunism even further. The Gram-negative anaerobes of the human body can appear in a variety of guises. They can be beneficial, as is the case with the colonic fermentation and the ability of colonic anaerobes to compete with and suppress pathogens. They can act as pathogens if they escape the area in which they normally reside. In the case of both the colonic and oral anaerobes, this type of infection is often seen as abscesses, probably due to the protection the bacteria gain if they lodge in anoxic areas of damaged tissue. Production of hydrolytic enzymes is also a common trait and one that may contribute to tissue damage. Finally, the Gram-negative anaerobes have raised the specter of new forms of disease, such as microbiota shift diseases, or a contribution to disease caused by the sharing of antibiotic resistance genes.

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Outer Membrane, Gram-Negative Bacteria

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Defining Statement
Composition, Structure, and Functions of Outer Membrane
Biosynthesis and Assembly of Outer-Membrane Components
Periplasm
Functional Complexes Involving Multiple Components of the Cell Envelope
Mycobacterial Cell Envelope
Further Reading

Glossary

β-barrel A protein-folding pattern in which an extensive β-sheet structure closes upon itself, producing a cylinder or a barrel structure.

bile salts Amphiphilic derivative of cholesterol secreted in bile and functioning as detergents for the digestion of fat in the intestinal tract. Conjugated bile acids often have strongly acidic groups (for example, from taurine).

Kdo An acidic, eight-carbon sugar 2-keto-3-deoxy-octonate or 3-deoxy-D-mannooctulosonate.

lipid bilayer An ordered two-layered arrangement of polar lipids so that their lipophilic portions associate in the center and their polar head groups face the outside.

lipopolysaccharide (LPS) A complex polymer comprising the outer leaflet of the outer-membrane bilayer. Its polysaccharide portion acts as a major antigen, and its lipid portion (lipid A) is responsible for its endotoxic activity.

molecular chaperone A protein that helps the folding of nascent proteins by binding to unfolded or misfolded polypeptides.

peptidoglycan (murein) An extensively cross-linked structure unique to bacteria, preventing the bursting of the cytoplasm by acting as a mechanically strong cage.

periplasm The space between the cytoplasmic membrane and outer membrane, containing peptidoglycan, membrane-derived oligosaccharides (MDO), and many unique proteins.

porin An outer-membrane protein that allows the nonspecific transmembrane diffusion of small, hydrophilic solutes.

R LPS An incomplete lipopolysaccharide containing only lipid A and the core portion of the saccharide chain, devoid of the O-chain. So-named because it is produced by mutants that show a 'rough'(R) colony morphology.

SecYEG A multiprotein export apparatus in bacteria, responsible for the transmembrane export of most proteins, including periplasmic and outer-membrane proteins.

sigma factor A subunit of bacterial RNA polymerase that recognizes a specific set of promoter sequences.

Toll-like receptor Usually transmembrane receptors on animal cell surface, which recognize various common components of microbial pathogens. So-named because of its similarity to the Toll receptor in *Drosophila*.

two-component system A signal transduction pathway widespread in bacteria, which consists of a histidine kinase sensor and a response regulator that becomes activated by the phosphorylation of its aspartate residue(s).

Abbreviations

ABC	ATP-binding cassette
ECA	enterobacterial common antigen
Kdo	ketodeoxyoctonate
LPS	lipopolysaccharide
MDO	membrane-derived oligosaccharides
RND	resistance-nodulation-division

Defining Statement

The cell envelope of Gram-negative bacteria contains, in addition to an inner, cytoplasmic membrane, an outer membrane – a structure unique to organisms of this group. The space between the two membranes is the periplasm, where we find not only the peptidoglycan (murein) cell wall but also many unique proteins. The outer membrane acts as a protective barrier, limiting the access of noxious compounds present in the environment.

The enteric bacteria, especially *Escherichia coli* and *Salmonella*, served as a paradigm for the studies of structure and function of the outer membrane.

Composition, Structure, and Functions of Outer Membrane

Lipids and Lipopolysaccharides

Outer membrane is distinctly different from the inner one or cytoplasmic membrane in terms of composition. The lipid bilayer domain, which is composed of mainly glycerophospholipids (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin in the enteric bacteria) in the inner membrane, additionally contains lipopolysaccharide (LPS) – a characteristic component. LPS is composed of two domains – the lipophilic lipid A domain and the hydrophilic polysaccharide domain. A single lipid A domain contains 5-7 fatty acid residues, all saturated and usually containing a 3-hydroxyl group, in contrast to just two often unsaturated fatty acid residues found in phosphatidylethanolamine or phosphatidylglycerol (**Figure 1**). Its head group is based on a phosphorylated glucosamine disaccharide, in contrast to the glycerol phosphate moiety in the glycerophospholipids. The lipid A head group is also connected to a polysaccharide structure, which protrudes into the external medium and plays a major role in the interaction of bacteria with the external world.

In *E. coli* and *Salmonella*, the entire outer leaflet of the bilayer appears to be composed of LPS, the phospholipids occupying only the inner leaflet (**Figure 2**). This asymmetric structure produces an exceptionally impermeable bilayer. Lipid bilayers can usually be penetrated easily by lipophilic molecules, including various drugs, because these molecules can dissolve into the interior of the membrane and redissolve in the aqueous phase on the other side of the membrane. However, the rate of such permeation is about two orders of magnitude slower across the outer-membrane bilayer. This is caused by the very low fluidity of the interior of the LPS leaflet, which in turn is the result of the presence of only saturated fatty acids, of many fatty acid chains connected covalently to a single head group, and of the bridging of neighboring, negatively charged LPS molecules by divalent cations (see below). Thus, the LPS and the LPS-containing outer leaflet of the outer membrane play a major role in producing an effective permeability barrier against the entry of lipophilic compounds, including many antibiotics. Penicillin G, isoxazolyl penicillins (such as oxacillin), erythromycin, and rifamycins show little activity against Gram-negative bacteria but strong

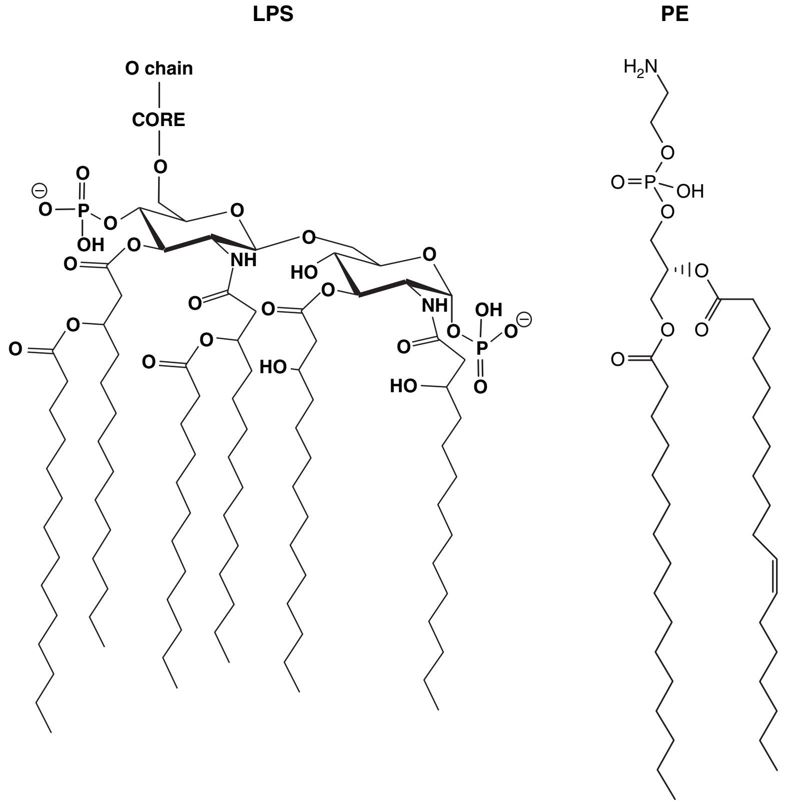

Figure 1 Structure of lipid A and phosphatidylethanolamine. For the latter, the most abundant species in *Escherichia coli* containing palmitate ($C_{16:0}$) and *cis*-vaccenate ($C_{18:1}$) at 1- and 2-position, respectively, is shown.

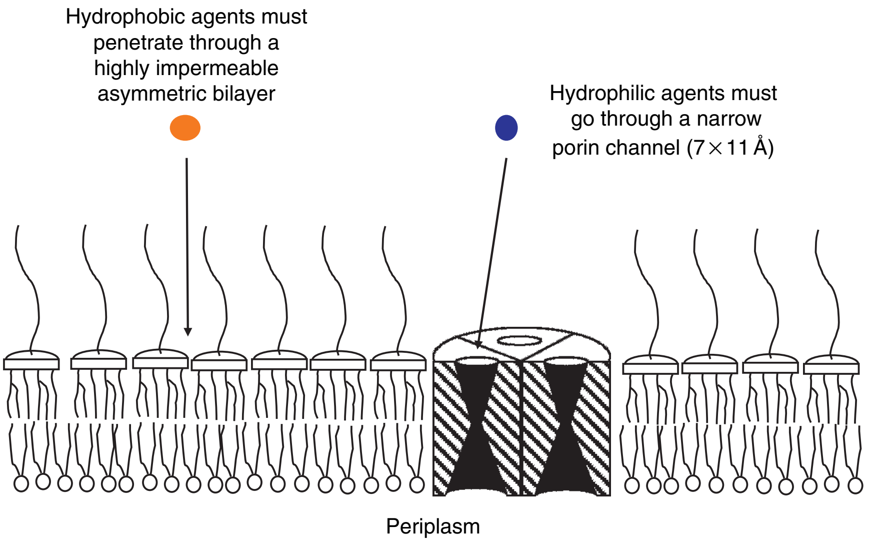
Hydrophobic agents must penetrate through a highly impermeable asymmetric bilayer
Hydrophilic agents must go through a narrow porin channel (7 × 11 Å)
Periplasm

Figure 2 A model of the outer membrane of enteric bacteria. In the bilayer domain, the outer leaflet is composed exclusively of lipopolysaccharides and the inner leaflet of glycerophospholipids. A nonspecific porin is also shown.

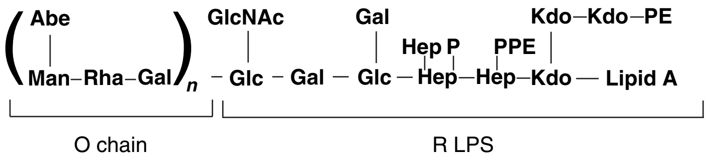

Figure 3 Structure of the polysaccharide portion of LPS. The figure shows, as an example, the LPS of *Salmonella typhimurium* (*Salmonella enterica* serovar Typhimurium). In this species, the O-chain is composed of a repeating unit, containing a 3,6-dideoxyhexose, abequose. Some of the residues in the R-LPS may not be present in stoichiometric amounts. Abe, abequose; Rha, L-rhamnose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Hep, L-*glycero*-D-*manno*heptose; Kdo, 3-deoxy-D-*manno*octulosonate; P, phosphate; P(PE), (pyro)phosphorylethanolamine.

activity against Gram-positive bacteria; this limited range is to a large extent due to the low permeability of the LPS leaflet.

The polysaccharide portion of LPS (**Figure 3**) is usually divided into the ‘core’, which is connected to lipid A, and ‘O side-chain’, which in turn is connected to the core. The former is composed of a nonrepeating oligosaccharide structure, which contains numerous acidic groups especially in its inner portion, close to the head group of lipid A. The glucosamine disaccharide head group also carries phosphate or pyrophosphate groups at both ends. Thus, the inner portion of LPS core has a very high density of negative charges, which are usually neutralized by divalent cations, such as Mg^{2+} and Ca^{2+}, in living bacterial cells.

The O-chain is most often composed of an oligosaccharide repeat unit, which frequently contains unusual sugars like 3,6-dideoxyhexoses. Since this is the portion exposed to the external world, animal pathogens will induce the production of antibodies that bind this portion of LPS. If the repeating unit contains unusual sugars, this may help the bacteria to evade the antibodies prevailing in the population of higher vertebrates. The structure of O-chain has thus undergone extensive modifications during recent evolutionary history so that serological analysis has shown the presence of more than 100 types of O-chains (recognized as ‘O antigens’) in *E. coli* alone.

Our body recognizes the lipid A domain of LPS as a characteristic common component of a pathogen through the Toll-like receptor on the surface of many cells, and this results in the strong stimulation of specific immune response that follows. Thus, this reaction is meant to help protect our body from infection, and some successful human pathogens are known to modify the lipid A structure to avoid the recognition by the host. Yet the recognition of lipid A domain by the Toll-like receptor can result in the overstimulation of many cells in our body, when many molecules of LPS are suddenly introduced, for example, by injection. Because of this reaction, LPS is also known as ‘endotoxin’. Humans and rabbits are some of the most susceptible animals to endotoxin; even 1 ng $(\text{kg body weight})^{-1}$ will cause significant increase in body temperature. A large amount of LPS is released into blood stream from dying bacteria, when patients suffering from sepsis (multiplication of bacteria in the blood stream) are treated by an effective antibiotic. This often causes severe, often fatal shock reactions (septic shock) in patients.

In addition to LPS, the outer membrane of the enteric bacteria contains enterobacterial common antigen (ECA). Unlike the O antigen polysaccharides, there is no variation in the structure among strains or even species, and all ECA molecules are composed of polysaccharides of *N*-acetylaminomannuronic acid, *N*-acetylfucosamine, and *N,O*-diacetylglucosamine. In the common form of this component, the polysaccharide is anchored to the outer membrane through a covalent linkage to phosphatidic acid.

The inner leaflet of the outer-membrane bilayer is composed of the common glycerophospholipids. However, the quantitative composition is different from that found in the inner membrane. Phosphatidylethanolamine occupies more than 90% of the glycerophospholipids in the outer membrane of *Salmonella*.

Proteins

The outer membrane is high in protein content. Several different classes of proteins are found.

Structural proteins

There is a need to anchor the outer membrane to the underlying peptidoglycan for stabilization (**Figure 4**). In enteric bacteria, this function is carried out by the murein lipoprotein (or Braun lipoprotein), which is a small periplasmic protein (7200 Da) with a covalently linked lipid moiety (a diglyceride and a fatty acyl residue are connected to the sulfydryl group and the amino group, respectively, of the N-terminal cysteine). This protein connects the two structures by becoming covalently linked to peptidoglycan at its C-terminus and noncovalently linked to the outer-membrane bilayer through its lipid extensions at its N-terminus.

In *Pseudomonas aeruginosa* and related bacteria, murein lipoprotein is absent. Instead, the outer membrane is connected to the peptidoglycan by OprF and its homologs. OprF is a homolog of *E. coli* OmpA, and most of this protein folds into a two-domain conformer with its N-terminal, eight-stranded β-barrel buried in the outer membrane; at the same time its C-terminal periplasmic domain associates noncovalently with the peptidoglycan (**Figure 4**).

Porins

Bacterial cells must take up nutrients from the environment in spite of the presence of the outer-membrane permeability barrier. For this purpose, all Gram-negative bacteria apparently synthesize a special class of outer-membrane protein called porins, whose narrow internal channel allows the diffusion of only small, hydrophilic nutrients. In *E. coli*, porins, which are present in about 10^7 copies per cell, are the most abundant protein in the cell in terms of mass. *E. coli* porin channel has a dimension of $0.7 \times 1.0\,\mathrm{nm}$ at its narrowest point, and disaccharides barely pass through this channel. Larger compounds (as a rough yardstick larger than about 600 Da) are essentially excluded. Interestingly, lipophilic molecules are strongly retarded in their passage through this channel; this is apparently because at the narrowest point of the channel (the 'eyelet'), the presence of acidic and basic amino acid residues on opposing walls (**Figure 5**) creates a layer of highly ordered water molecules and passage of lipophilic solutes through this layer is energetically unfavorable as it will disrupt this structure. Finally, *E. coli* porins favor the passage of cations over anions. Note that these properties of the porin channels serve to prevent the entry of many toxic compounds, for example, conjugated bile salts (lipophilic and anionic), perhaps the

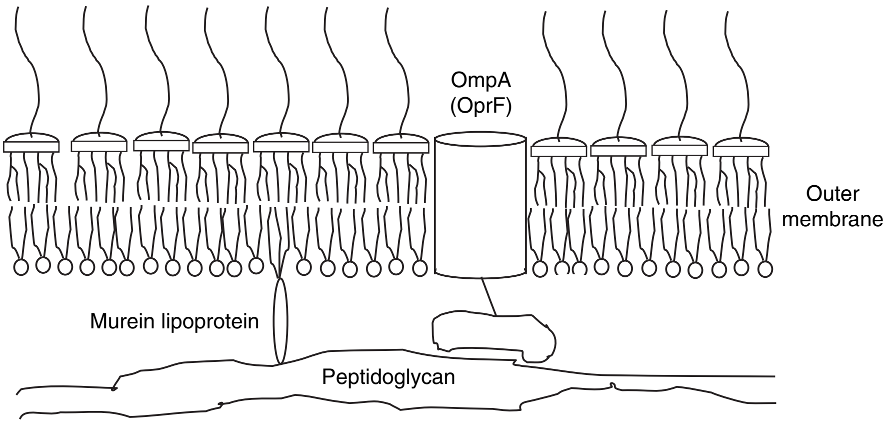

Figure 4 Anchoring of outer membrane to the peptidoglycan layer.

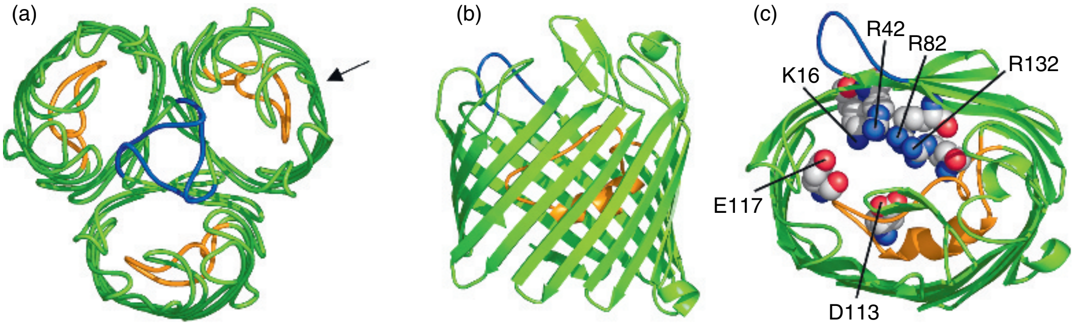

Figure 5 X-ray structure of *Escherichia coli* OmpF porin. (a) View of the trimer from the top. Loop 2, colored blue, connects one protomer to the neighboring protomer. Loop 3, colored orange, folds back into the lumen of the β-barrel and narrows the diffusion channel. (b) View of the monomeric unit from the side. (c) View of the monomeric unit from the top, showing the 'eyelet' or the constricted region of the channel. The eyelet is formed by acidic residues Glu117 and Asp113 from the Loop 3, and basic residues from the opposing barrel wall, Lys16, Arg42, Arg82, and Arg132, all shown in spherical models. Reproduced from Nikaido H (2003) Molecular basis of bacterial outer membrane revisited. *Microbiology and Molecular Biology Reviews* 67: 593–656, with permission from the American Society for Microbiology.

most important inhibitor for *E. coli* living in the intestinal tract of higher animals.

E. coli porins are trimers, each of which is composed of 16-stranded β-barrels (**Figure 5**). *E. coli* produces three porins: OmpF, OmpC, and PhoE. Their structures are very similar to each other, yet there are subtle differences especially within the channel. In contrast to OmpF and OmpC, PhoE favors the permeation of anions and is expressed only under phosphorus starvation conditions presumably to enhance the uptake of phosphate and its esters from the environment. OmpC behaves as though its channel is slightly narrower than that of OmpF and becomes a predominant porin when the ionic strength of the environment is close to, or over, that in the body fluids of higher animals. The predominance of OmpC in *E. coli* living in the intestinal tract will further enhance the exclusion of bile acids.

It should be emphasized that porins discriminate among various solutes based on their global physicochemical properties, such as size, charge, and lipophilicity, and in this sense they show no specificity based on the exact fit between the protein and the ligand. Thus PhoE, for example, favor the diffusion of any anions such as sulfate or anionic antibiotics, although such a behavior is not beneficial for the *E. coli* cells starved for phosphorus.

Although trimeric porins are found universally in enteric bacteria, they are often absent in other groups of Gram-negative bacteria. For example, *P. aeruginosa* and its relatives produce very impermeable outer membranes; they do not contain close homologs of OmpF/OmpC/PhoE, and the rate of penetration of hydrophilic solutes (such as cephalosporins) is almost two orders of magnitude slower than across the *E. coli* outer membrane. The major nonspecific porin of these species is OprF, which is a homolog of *E. coli* OmpA, and similarly acts as a structural protein anchoring the outer membrane to the peptidoglycan. The rate of penetration of solutes through the OprF channel is indeed nearly two orders of magnitude slower than through the OmpF channel, and this is explained by the fact that only a few percent of OprF protein folds to produce an open channel, although the channel itself is not narrow, with an estimated diameter of about 2.0 nm.

Specific channels

It would be beneficial for *E. coli* to take up some solutes rapidly that are retarded or excluded by the trimeric porins. For *P. aeruginosa* with its inefficient general purpose porins, the need to take up various types of nutrients more efficiently is acute. For these purposes, many Gram-negative bacteria produce specific diffusion channels. Since starch (amylose) is broken down to oligosaccharides of maltose series by pancreatic amylase in our intestinal tract, *E. coli* must take up these compounds efficiently. For this purpose, it uses the LamB (lambda B, so named because it is used as the receptor by the *E. coli* bacteriophage lambda) channel, which served as an example of these specific channels.

LamB is also a trimeric protein composed of β-barrels. The barrel is slightly larger than in the porin, containing 18 strands in contrast to 16 in the OmpF homologs. However, the channel is narrower. The interior of the channel is exquisitely constructed to facilitate the passage of maltose oligosaccharides, with the succession of six aromatic amino acid residues constituting a 'greasy slide' on which the sugars can slide past (**Figure 6**). The channel has affinity to maltose and other higher oligosaccharides of this series, and this serves to accelerate the transmembrane diffusion of these sugars (up to maltohexaose, 990 Da) when they are present in low concentrations. When they are present in

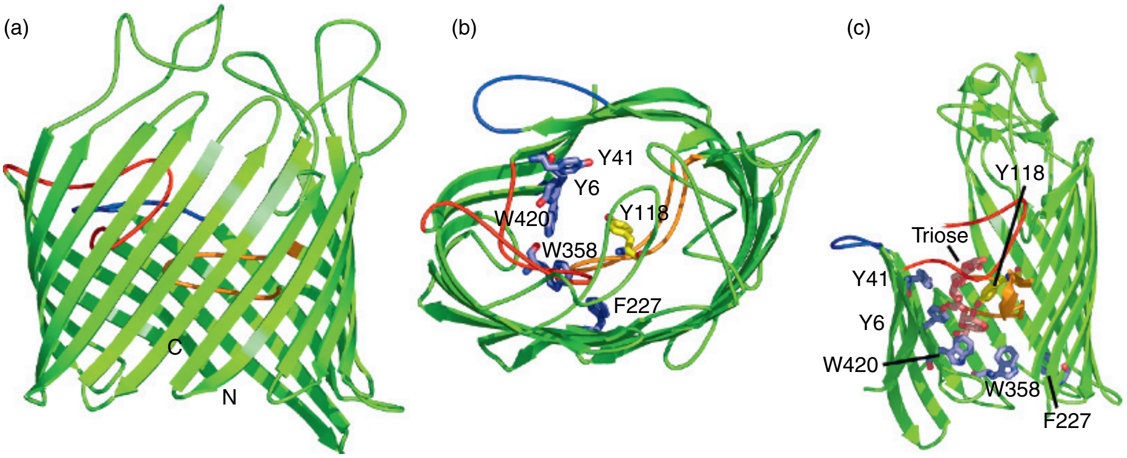

Figure 6 X-ray crystallographic structure of a specific channel, *Escherichia coli* LamB. (a) Side view of the monomeric unit. Loop 3 (orange) and loop 1 (red) between the transmembrane β-strands (shown as flat arrows) fold back deeply into the central channel of the β-barrel, and makes the channel narrower. Loop 2 (blue) folds outward and interacts with other protomers of the trimeric structure. (b) View of the monomeric unit from the top. The residues of the greasy slide (Tyr41, Tyr6, Trp420, Trp358, and Phe227) are shown as blue stick diagrams, and Tyr118, which constricts the channel from the other side, is shown as a yellow stick diagram. (c) View of the greasy slide and its interaction with maltotriose. This is a side view, with the front of the β-barrel cut out for a better view of the slide. The aromatic residues comprising the helically twisted slide and Tyr118 are shown as stick diagrams, colored as in (b). The maltotriose molecule (Triose) is shown as a stick diagram colored in orange. The diagrams are based on PDB coordinate files 1MAL and 1MPN, and produced with PyMol. Reproduced from Nikaido H (2003) Molecular basis of bacterial outer membrane revisited. *Microbiology and Molecular Biology Reviews* 67: 593–656, with permission from the American Society for Microbiology.

nonphysiological, high concentrations, smaller oligosaccharides such as maltose can diffuse through the nonspecific porin channels as well, or even faster.

There are several other specific *E. coli* channels that have been studied in detail. These include the channels for sucrose (ScrY), arylglucosides (BglH), nucleosides (Tsx), and fatty acids (FadL). In *P. aeruginosa*, the channel protein OprD was studied because it plays a major role in the rapid influx of imipenem, an antibiotic that shows an exceptional activity against this organism, and appears to be a specific channel for basic amino acids and peptides. Interestingly, *P. aeruginosa* contains 19 homologs of this protein; probably each of these produces a specific diffusion channel for a specific type of nutrients needed by this organism, whose general purpose porin, OprF, is very inefficient as described above.

TonB-dependent receptors

Gram-negative bacteria need to take up some large compounds that exist in extremely low concentrations in the environment. These include the iron–siderophore complexes for the uptake of iron and vitamin B_{12}. *E. coli*, for example, produces specific outer-membrane transporters for ferric enterobactin (726 Da, the chelator produced by *E. coli* itself) (FepA), ferrichrome (chelator produced mostly by fungus) (FhuA), coprogen (FhuE), ferric citrate (FecA), dihydroxybenzoate–ferric complex (Fiu), ferric complexes of similar catecholate-type compounds (Cir), and vitamin B_{12} (1355 Da) (BtuB). For the uptake of these compounds, even specific channels are not adequate because diffusion through these channels is driven ultimately by the difference in concentrations across the membrane, which is vanishingly small for these compounds. They are, therefore, transported across the outer membrane by an active process with the concomitant consumption of energy. Because there is no ATP in the periplasm or in the outer membrane, the energy coupling is done by an ingenious process of using a periplasmic protein, TonB, to transduce the energy from the cytoplasmic membrane, as discussed in 'Functional complexes involving multiple components of the cell envelope'.

The outer-membrane proteins involved are usually called receptors because they show very strong affinity to the transported ligands, with the dissociation constants in the range of nanomolar. Since the ligands are present in such low concentrations, this high affinity is absolutely needed. X-ray crystallographic studies showed that the proteins have the basic construction of a β-barrel, with 22 transmembrane β-strands (**Figure** 7). The proteins appear to exist as monomers, in contrast to the OmpF-type porin and the LamB protein. Furthermore, a large N-terminal portion is folded into the barrel, as though to 'plug' the large central channel inside the β-barrel. Recent years have seen impressive progress in our understanding of the interaction between TonB and these receptors (see below), but we still do not know how the active transport of the ligands is achieved.

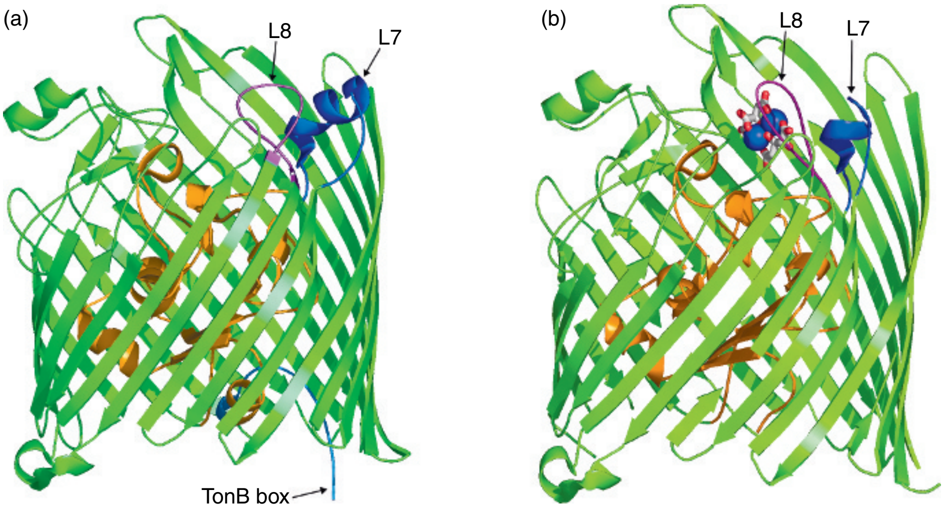

Figure 7 X-ray structure of the ferric citrate receptor, FecA. (a) Side view of the unliganded FecA. The N-terminal domain of this protein is inserted into the lumen of the large β-barrel (green) as a 'plug' (orange). Its N-terminal end contains the 'TonB box' sequence, predicted, and now shown, to interact with the C-terminal part of the TonB protein. Loops 7 and 8 are shown in deep blue and mauve, respectively. (b) Liganded FecA. Binding of ferric citrate (shown with two large blue spheres indicating iron atoms and with stick models for citrate) produces movement of loops 7 and 8. The TonB box also becomes disordered and invisible. Based on PDB files 1KMO and 1KMP. Reproduced from Nikaido H (2003) Molecular basis of bacterial outer membrane revisited. *Microbiology and Molecular Biology Reviews* 67: 593–656, with permission from the American Society for Microbiology.

Export channels

E. coli TolC is an outer-membrane protein that exists as a symmetrical trimer. The trimer contains the 12-stranded β-barrel that functions as a channel traversing the outer membrane, but in this case each monomer contributes only 4 of the 12 transmembrane strands. Remarkably, the channel extends about 10 nm into the periplasm, this time as a bundle of 12 long α-helices. This arrangement allows the end of the periplasmic α-tunnel to become connected to the external end of the transporters in the inner membrane, producing a direct extrusion mechanism for substrates into the medium, bypassing the periplasm. Thus, TolC and its homologs are essential components for some of the machineries that export proteins and drugs (see below).

A very different arrangement is also used in the export of various proteins into the medium. Here a rather large number (12–14) of intrinsic outer-membrane proteins assemble to produce a large central channel. This occurs in the Type II secretion pathway (discussed below) and the protein here is called *secretins*. Similar large channels are also envisaged for the pathway leading to the export of P pili subunits, and here the proteins are called *ushers*.

Biosynthesis and Assembly of Outer-Membrane Components

Biosynthesis of Component Macromolecules

The synthesis of macromolecules (LPS, outer-membrane proteins) obviously takes place in the cytosol where ATP and other sources of energy are available. The biosynthesis of LPS is now understood in details. The synthesis of lipid A starts from an activated form of *N*-acetylglucosamine, UDP-*N*-acetylglucosamine (**Figure 8**). In the first reaction, a C_{14} hydroxylated saturated fatty acid (3-OH myristic acid) residue is added to the 3-position of the sugar (step 1 in **Figure 8**), followed by the deacetylation (step 2) and further addition of another 3-OH myristic acid residue on the now-deacetylated 2-amino group on the sugar (step 3). Two of these diacylated molecules are fused together to produce a 1-phosphorylated tetraacylated disaccharide (step 4), which is then phosphorylated at the other end of the disaccharide (step 5) to produce a structure commonly called Lipid IV_A, which shows significant endotoxic activity. Just like the acylation reactions preceded the formation of disaccharide, the addition of the innermost sugar residues, two Kdo (ketodeoxyoctonate, or 3-deoxy-D-mannooctulosonic acid) residues of the core portion are added next (step 6) before the addition of two other fatty acid residues to complete the acylation reactions (steps 7 and 8).

The product of the series of reactions described above $(Kdo)_2$-lipid A, then accepts various sugars of the core domain (**Figure 3**) from nucleotide-sugar donor molecules. Since these nucleotide sugars are components of the cytosol, these transfer reactions must occur at the inner surface of the cytoplasmic membrane. The final product of the synthesis up to this point, the complete R LPS (see Glossary), is flipped across the cytoplasmic membrane by an ATP-binding cassette (ABC) transporter MsbA.

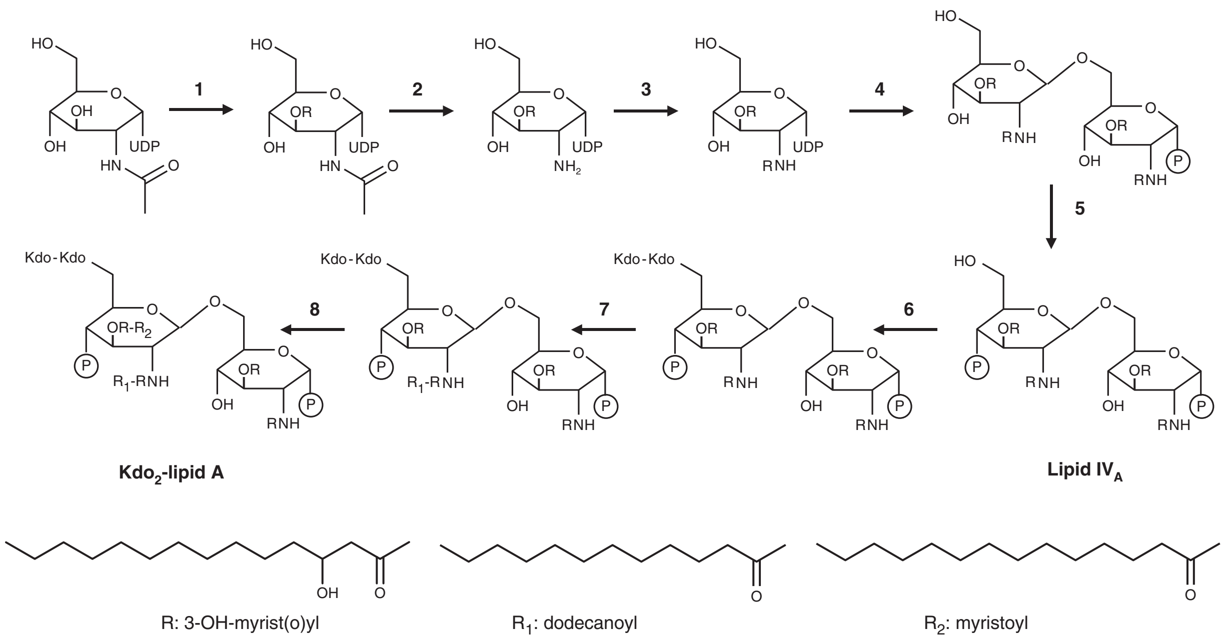

Figure 8 Pathway of lipid A biosynthesis. Note that acylation of the sugar occurs before the formation of the disaccharide head group, and that addition of two Kdo residues occurs before the completion of all acylation reactions. Thus, the final product is $(Kdo)_2$-lipid A and is not lipid A (which was defined as a product of partial acid hydrolysis of LPS).

The biosynthesis of the outer part of the LPS polysaccharide, O-chain, proceeds separately from that of the R LPS. Many O-chains are composed of branched oligosaccharide repeat units, and such a unit is assembled again at the inner surface of the cytoplasmic membrane by using nucleotide-sugar molecules as donors on a large (C_{55}) isoprenoid lipid carrier, undecaprenol phosphate, bound noncovalently to the membrane. When the repeating unit assembly is complete, the oligosaccharide–lipid complex is apparently flipped over across the membrane by a transporter called Wzx. At the outer surface of the membrane, the repeating units are polymerized by an enzyme Wzy, and the completed O-chain is finally transferred, at the periplasmic surface of the cytoplasmic membrane, to the nascent R LPS.

Some unbranched O-chains of rather simpler structure are assembled again on the inner surface of the cytoplasmic membrane. However in this case, the completely assembled, long O-chain is extruded by an ABC transporter across the cytoplasmic membrane before its transfer to the R LPS presumably at the outer surface of the cytoplasmic membrane.

Outer-membrane proteins are synthesized by ribosomes in the cytosol. They are then exported across the cytoplasmic membrane, apparently always utilizing the SecYEG pathway. How these proteins become assembled into the outer membrane is described in the next section.

Assembly

The components of the outer membrane, after their export across the cytoplasmic membrane, somehow travel across the periplasm and are finally assembled in the outer membrane. After the export across the cytoplasmic membrane, all the processes must proceed without utilizing ATP. Some of the mechanisms involved are becoming elucidated in the last several years.

Assembly of outer-membrane proteins

The export of lipoproteins is understood in most detail. After the apoprotein is exported in the unfolded state by the SecYEG machinery, the lipids are added. Although this is done on the periplasmic surface of the cytoplasmic membrane where no ATP is available, the transfer is done by utilizing membrane phospholipids as donors. It was known for some time that some lipoproteins associate themselves with the inner leaflet of the outer membrane, whereas others remain anchored to the outer leaflet of the inner membrane; furthermore the fate of the lipoproteins depends on the nature of the second amino acid residue from the N-terminus after the cleavage of the leader sequence. If this residue is aspartate, the protein is retained in the inner membrane, and if it is a neutral residue such as serine, the protein is exported to the outer membrane.

Recent studies elucidated the molecular mechanism of this differential export process for lipoproteins. Thus, lipoproteins become the substrates of an unusual ABC transporter, LolCDE, whose function is to 'transport' them (with the utilization of ATP at the inner surface of the cytoplasmic membrane) to a periplasmic-binding protein, LolA (**Figure 9**). LolA–lipoprotein complex then reacts with the LolB protein, located at the inner surface of the membrane, and the lipoprotein is now delivered into the inner leaflet. Those lipoproteins with aspartate as the second amino acid will escape the recognition by the LolCDE complex, and will thus remain anchored in the cytoplasmic membrane.

We have also gained some understanding on the mechanism of assembly of the integral outer-membrane proteins. Unlike lipoproteins that are inserted into the outer membrane only through their lipid groups, most integral outer-membrane proteins traverse both leaflets of the membrane and characteristically are composed of β-barrels. Since most intrinsic proteins of the cytoplasmic membrane are bundles of transmembrane α-helices, the unusual β-barrel conformation of the outer-membrane proteins certainly suggests a unique mechanism of export and assembly for these proteins. Recently, an outer-membrane protein, called Omp85 (or YaeT), was found to be at the center of this assembly process. In a cell depleted for Omp85, outer-membrane proteins accumulate in an unfolded form and not into the outer membrane. Omp85 is also a β-barrel, but it has a long N-terminal extension containing repeated sequences that are postulated to play a chaperone-like role in binding to incompletely folded proteins. Omp85 exists in association with several proteins, and this protein complex is likely to interact with outer-membrane proteins in an incompletely folded state and to help them to fold correctly and become inserted properly into the outer membrane. The presence of Omp85 homologs in the outer membranes of chloroplasts and mitochondria and their apparent role in the assembly of proteins in these organellar outer membranes support the important role for this protein.

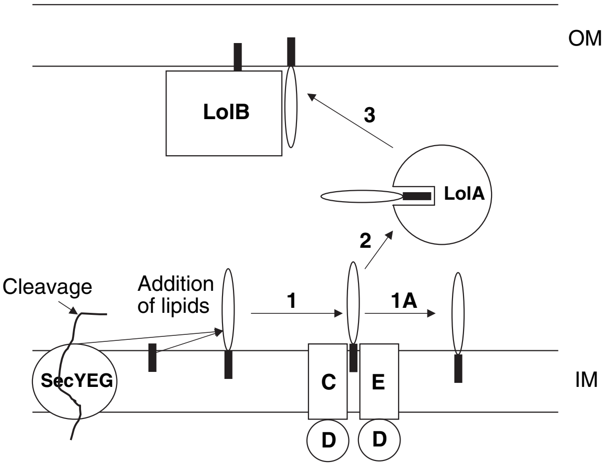

Figure 9 Mechanism of lipoprotein export. After export through the SecYEG exporter, the signal sequence is cleaved to expose the N-terminal cysteine of the mature protein. This residue is lipid-modified, and the lipoprotein interacts with the LolCED ABC transporter, with its ATPase units (LolD) (step 1). If the second amino acid residue is Asp, it is an inner-membrane lipoprotein and is released and stays in the inner membrane (step 1A). If it is a neutral amino acid, then the lipoprotein is actively transferred to a periplasmic binding protein, LolA (step 2), and finally interacts with the outer-membrane receptor protein LolB (which itself is a lipoprotein), to be assembled into the inner leaflet of the outer membrane (step 3).

Since proteins are secreted through the SecYEG apparatus in their unfolded form, they will aggregate as denatured proteins in the periplasm before their interaction with Omp85 complex in the outer membrane, if no protective mechanisms exist. Indeed, periplasm is known to contain many proteins that would act as chaperones and prevent irreversible denaturation of the outer-membrane proteins in transit. Skp, a small, basic periplasmic protein, and SurA, a periplasmic chaperone that also has an activity in catalyzing the *cis–trans* interconversion of peptidyl-proline isomers, are thought by some workers to play an especially important role in this process.

The process of export and assembly of outer-membrane proteins is thus complex, and errors are likely to occur at every step of the pathway. Thus, the periplasm has a highly evolved mechanism for sensing such errors and for correcting them (see 'Periplasm').

Assembly of LPS

We have described how the complete LPS is assembled on the outer (periplasmic) surface of the cytoplasmic membrane. LPS, however, has still to cross the periplasm, become inserted into the outer membrane, and most importantly cross the bilayer to reach its final location in the outer leaflet of the outer membrane. How this complex series of reactions takes place is not yet clear. Recently, however, one outer-membrane protein apparently involved in this process has been identified. This protein, called Imp (for increased membrane permeability), was known as an essential protein in *E. coli*, and its mutated version was known to produce *E. coli* cells with a 'leaky' outer membrane showing an imperfect barrier properties. LPS is also essential for the survival of *E. coli*. In contrast, another species of Gram-negative bacteria, *Neisseria meningitidis*, can survive without LPS. Imp is also not essential in this organism. Inactivation of the *imp* gene in this organism showed that none of the newly synthesized LPS appeared on the cell surface, that is, in the outer leaflet of the outer membrane, suggesting the role of Imp in the correct assembly of LPS in its proper location. The molecular mechanisms involved, however, are yet not clear.

Assembly of phospholipids

Phospholipids are synthesized in the inner leaflet of the inner membrane, and thus the first step for its export is expected to be the flipping to the outer leaflet. Although MsbA was suggested to catalyze this reaction, in *Neisseria msbA* null mutants export and assemble phospholipids into the outer membrane, and thus MsbA cannot be essential for phospholipid export. Currently, there is no information on the export pathway of phospholipids, which were shown to equilibrate rapidly between the outer and inner membranes.

Periplasm

The space between the outer and inner membranes contains the periplasm. This is not just an empty 'space', and it contains many distinct components that carry out important functions. Obviously, this space contains peptidoglycan, but hints of its unique function came from the discovery that several degradative enzymes of *E. coli*, for example ribonuclease and alkaline phosphatase, are found exclusively in the periplasm. Since these enzymes will destroy essential components of the cytosol if they existed in the cytoplasm, their compartmentalization within the periplasm, outside the cytosol, makes sense in terms of physiology.

One analysis on the basis of genome sequence of *E. coli* predicts about 370 periplasmic proteins, in comparison with only about 150 proteins to be located in the outer membrane. Another large class of periplasmic proteins includes ligand-binding proteins that form a part of the ABC transporters that import many compounds, including sugars, amino acids, peptides, inorganic ions such as phosphate and sulfate as well as metal cations, ferric–siderophore complexes, vitamin B_{12}, and others. Another class includes proteins involved in electron transport. Now it is known that enzymes like formic hydrogenlyase and aerobic nitrate reductase, with their molybdate factor, are components of the periplasm. Although *E. coli* lacks the typical cytochrome *c* found in the corresponding space in mitochondria, there are several *c*-type cytochromes that function in the reduction of alternate electron acceptors. Yet other important class includes enzymes involved in peptidoglycan biosynthesis. β-Lactamase, which was apparently derived from one of these enzymes during evolution, plays a predominant role in raising the intrinsic level of resistance of Gram-negative bacteria to penicillins and cephalosporins, the most important class of antimicrobial compounds. Finally, periplasm contains many proteins that assist in the folding of periplasmic and outer-membrane proteins. These include proteins involved in the formation of disulfide bonds, such as DsbA, DsbB, DsbC, and others. The cytoplasm of Gram-negative bacteria is a highly reducing environment, which contains hardly any disulfide-bond-containing protein. In contrast, disulfide bonds are found commonly in periplasmic and outer-membrane proteins, and their formation presumably helps in the folding of these proteins after their export, in an unfolded form, through the SecYEG export apparatus across the cytoplasmic membrane. Periplasm also contains many proteins that appear to function as molecular chaperones including Skp and SurA that have already been mentioned.

In addition, it is essential to have an intricate regulatory mechanism that senses the proper functioning of the folding machinery. In fact, periplasm contains at least two major such signal transduction pathways. One of them, sigma E pathway, becomes activated when periplasmic or outer-membrane proteins misfold at high temperature or under other conditions, and the activation of the alternative sigma factor E increases the transcription of many dozens of genes, many related to the folding of proteins in the periplasm, such as SurA, Skp, DsbC, and another peptidyl-prolyl isomerase, FkpA. At the same time, the synthesis of periplasmic proteases such as DegP is increased strongly, presumably to degrade misfolded proteins. The response also increases the transcription of many genes involved in lipid A synthesis, as well as the genes for Omp85 and Imp. The transcription of major outer-membrane protein genes (*ompF, ompC, ompA*) is on the other hand strongly repressed. The sigma factor works with the core RNA polymerase in the cytosol; yet it has to be activated by signals in the periplasm. Evidence suggests that sigma E factor is normally held captive by a transmembrane protein RseA, but the appearance of misfolded proteins in the periplasm activates a periplasmic protease DegS, which degrades RseA, ultimately resulting in the release of sigma E factor into the cytosol.

The second signal transduction pathway, CpxAR, is a typical two-component system that uses an intrinsic inner-membrane protein CpxA to sense the extracytosolic signal. It responds to the presence of misfolded proteins in the periplasm, but most interestingly it is stimulated by other signals, for example the attachment of *E. coli* cells to a hydrophobic surface. Thus, this system may sense some subtle changes in the external world.

The general physical environment of the periplasm requires some comment. It was shown by Stock and colleagues that the periplasm is in osmotic equilibrium with the cytosol. Cytosol is usually kept at a hyperosmotic state in comparison with the medium so that the cytosol will not collapse and a moderate outward pressure is constantly present at the cytoplasmic membrane in a 'balloon-in-a-cage' arrangement limited by the mechanically strong peptidoglycan. How could then the periplasm remain hyperosmotic in relation to the medium in the presence of so many porin channels? Stock and coworkers found that interior-negative Donnan potential exists across the outer membrane and this results in the passive accumulation of cations in periplasm. Later studies led to the identification of the cause of this Donnan potential – the presence in the periplasm of 'membrane-derived oligosaccharides' (MDO), a group of glucose oligosaccharides too large to escape through the porin channels and containing multiple negative charges. MDO is produced in large amounts when the osmotic activity of the medium is low so that much cations can be concentrated in the periplasm. Protons as cations are also concentrated in the periplasm. Thus depending on the conditions, the periplasmic pH can be significantly lower than that of the medium – a fact that is not generally appreciated. The Donnan potential also affects the uptake of drugs across the outer membrane, favoring that of cationic drugs (especially polycationic ones such as aminoglycosides) and preventing to some extent the uptake of anionic compounds.

Functional Complexes Involving Multiple Components of the Cell Envelope

In the late 1960s, Manfred Bayer found by electron microscopy structures that seemed to connect the outer membrane with the inner membrane. This discovery was greeted with skepticism at that time, but recent genetic and biochemical studies showed many instances where physical interaction must occur between the components of the two membranes.

An example involves the outer-membrane receptors for iron–siderophore complexes and vitamin B_{12}. Bacteria need iron as a component of many enzymes and respiratory complexes. However, the ferric ion found in aerobic environment occurs at an exceedingly low concentration because of its low solubility. Thus bacteria produce siderophores, or iron carriers, that bind the ferric ion strongly and move them into the cytoplasm. However, both iron–siderophore complexes (726 Da for ferric enterobactin) and vitamin B_{12} (1355 Da) are too large to pass through the porin channels of enteric bacteria. Thus these bacteria produce specific outer-membrane receptor proteins that bind these compounds strongly. Transport of these compounds into the periplasm is an energy-dependent process, driven by the proton-motive force across the inner membrane. The energy is transmitted from the inner membrane to the outer-membrane receptor by TonB, a periplasmic, elongated protein that is embedded in the cytoplasmic membrane at one end and appears to traverse the thickness of the entire periplasm to interact with the outer-membrane receptors. This process is thus likely to involve physical connection between the inner and outer membranes (**Figure 10**).

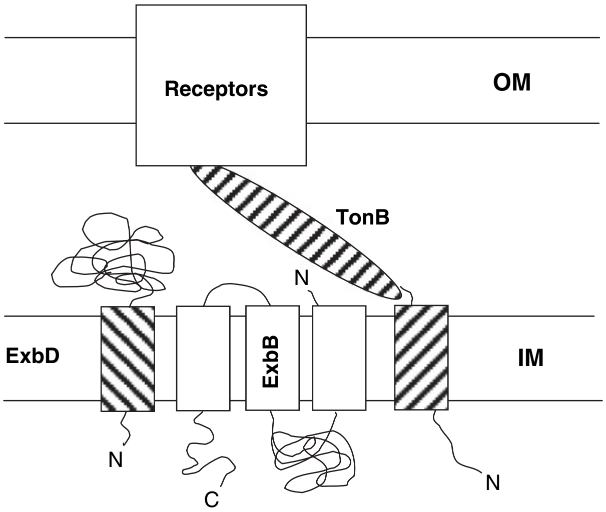

Figure 10 Hypothetical model of TonB-dependent transport complex. TonB appears to have an N-terminal transmembrane helix, which may interact with at least two other intrinsic inner-membrane proteins, ExbB and ExbD, needed for energy transduction. The other end of TonB has been hypothesized to interact with the TonB box of various receptors, and indeed cocrystals of receptors with TonB showed that this physical interaction occurs.

Another example is a group of multidrug efflux pumps in Gram-negative bacteria. These bacteria were found to pump out great many antibiotics and chemotherapeutic agents since mid-1990s, often utilizing transporters of an astonishing degree of versatility. One class, called resistance-nodulation-division (RND) superfamily, is particularly important. They are ubiquitous and often show an extremely wide range of substrate specificity. For example, AcrB (so named for acridine resistance) pump of *E. coli*, which served as the prototype of these transporters, can pump out not only most of the commonly used antibacterials (including penicillins, cephalosporins, macrolides, rifampicin, fluoroquinolones), dyes, antiseptics, and detergents but also even simple solvents. Furthermore, RND pumps are organized into multiprotein complexes that span both the inner and outer membranes (**Figure 11**), together with an outer-membrane channel (TolC in *E. coli*) and a periplasmic linker protein. This construction allows the complex to move drug molecules directly into the medium, a mechanism that is very advantageous in Gram-negative bacteria. A simple, uncomplexed pump located in the inner membrane would only move drugs from the cytosol to the periplasm, and the drug molecules can diffuse into the cytosol spontaneously and rather easily, as drugs with cytosolic targets must have rather lipophilic structures to allow this influx process. In contrast, the tripartite, cell envelope-spanning structures of the RND pumps (**Figure 11**) cause the movement of drugs from either

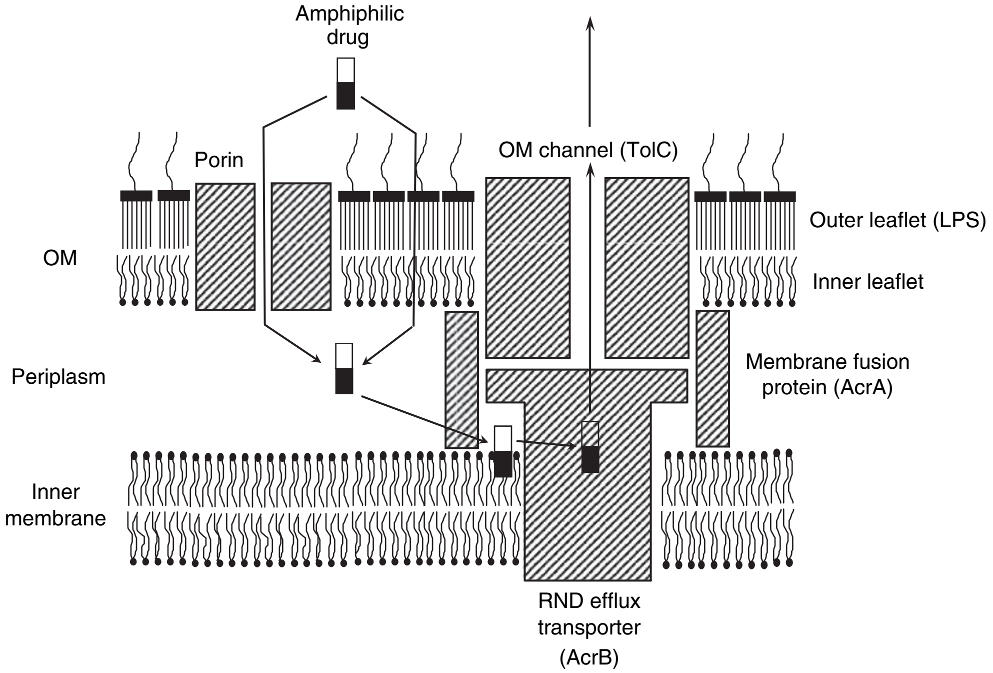

Figure 11 A model of RND-type efflux pump complex. RND transporters such as AcrB characteristically contain a very large periplasmic domain, which interacts with the end of the periplasmic extension of the TolC channel. The complex is further stabilized by a periplasmic linker protein or membrane fusion protein, in this case AcrA. Amphiphilic drugs slowly penetrate across the outer membrane, either through the porin channel or the asymmetric bilayer, and on reaching the periplasm are likely to become captured by the periplasmic domain of the transporter, to be expelled directly into the medium. X-ray crystallographic data exist for each of the components, but so far the whole complex has not been analyzed by crystallography.

the cytosol or the periplasm into the external medium; thus, the extruded drug molecule has to traverse the effective outer-membrane barrier again to come into the cell. It is well known that most of the naturally occurring antibiotics (e.g., penicillin G and erythromycin) and the library of chemically synthesized antibacterial compounds (e.g., linezolid) exhibit activity only against Gram-positive bacteria. This ‘intrinsic’ resistance of Gram-negative bacteria was often attributed to the barrier properties of the outer membrane. However in many cases, the inhibitors are actively pumped out by these ubiquitous multidrug efflux pumps, and the contribution of the pumps is at least as important as that of the outer-membrane barrier.

Final examples of the structures spanning both of the membranes are machineries dedicated for extracellular secretion of proteins and other macromolecules. With Gram-positive bacteria, protein secretion is only a matter of moving protein molecules across the cytoplasmic membrane. In contrast, with Gram-negative bacteria, the secreted protein must cross two barriers, and it becomes necessary to prevent the premature folding of the protein in the periplasm and to push the protein through another membrane barrier, the outer membrane. Yet most bacteria, especially pathogens, have a need to secrete toxic proteins into the external space, and in some cases even into the host cells, a process requiring the breaching of yet another membrane barrier.

Several mechanisms have been devised for protein secretion in Gram-negative bacteria (**Figure 12**). As seen, all these machineries involve protein assemblies that traverse the entire cell envelope. In the simplest system, the Type I secretion pathway, the proteins are pushed out across the cytoplasmic membrane by a transporter, a member of the ABC superfamily. Although ABC transporters catalyze the active transport (both import and export) of a variety of substrates, both small and large, this system is unusual in the sense that the transporter exists as a multiprotein complex including the outer-membrane-channel protein TolC and a periplasmic linker protein so that the exported protein can cross the entire cell envelope, without facing the aqueous environment of the periplasm. The system shown is utilized in the secretion of hemolysin, which is a toxin, by *E. coli.*

In the Type II secretion system (**Figure 12**), the protein is exported across the cytoplasmic membrane by the SecYEG complex, which is also responsible for the secretion of most proteins into the periplasm as well as in Gram-positive bacteria. However, again in this case many additional proteins appear to collaborate to produce the passage across the envelope. The movement of

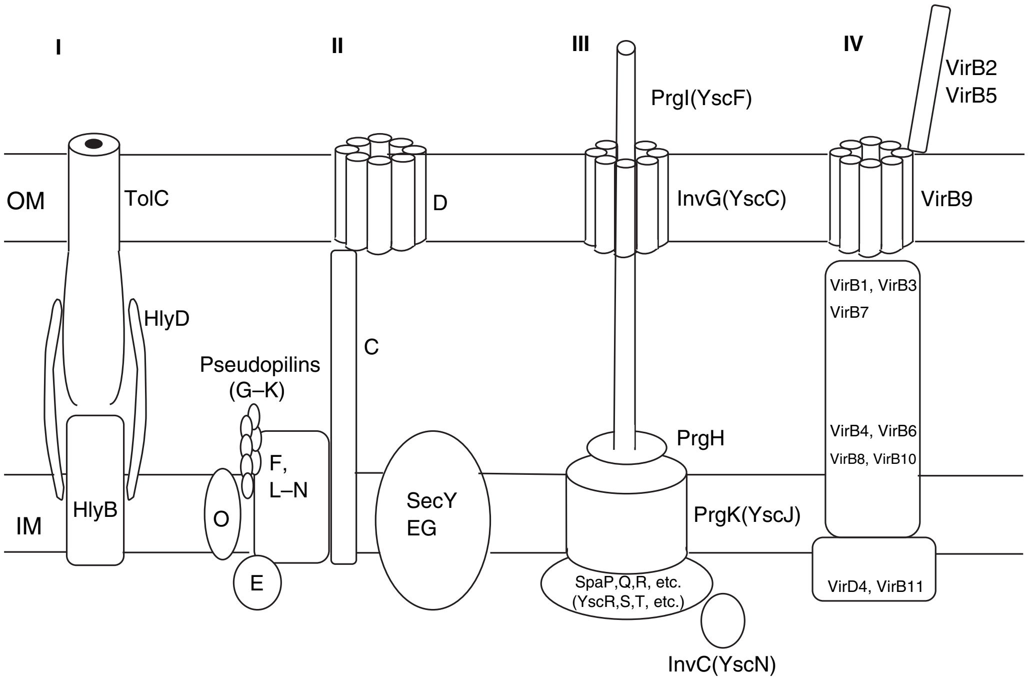

Figure 12 Machineries for protein export in Gram-negative bacteria. The figure shows the construction of Type I through Type IV protein secretion apparatus in a schematic manner. For the Type III system, nomenclature for the *S. typhimurium* system is shown, with that for the *Yersinia* system in parentheses. Reproduced from Nikaido H (2003) Molecular basis of bacterial outer membrane revisited. *Microbiology and Molecular Biology Reviews* 67: 593–656, with permission from the American Society for Microbiology.

the protein from the periplasm to the external medium requires energy, and this is thought to be supplied by the hydrolysis of ATP at the inner surface of the cytoplasmic membrane (by the protein 'E' in the figure). This is reminiscent of the energization of outer-membrane transport of siderophores and vitamin B_{12} by the proton-motive force, located in the inner membrane.

In the Type III secretion system (**Figure 12**), again a complex assembly of many proteins is required. This system usually has a long, needlelike extension outside and is often thought to 'inject' proteins into the cytosol of the host cells. Indeed, the genes coding for this system are often found in the 'pathogenicity islands' on the bacterial chromosome, where many genes involved in the infection process are clustered together. Another fascinating aspect of this system is that many of its proteins are similar to the components of the basal bodies of bacterial flagella, suggesting that flagella and this secretion system have a common evolutionary origin.

Type IV secretion system is also complex and contains many proteins (**Figure 12**). A prototype of this system is the VirB system (shown in **Figure 12**) that is responsible for the export of a piece of single-stranded DNA (and associated proteins) from the bacterium *Agrobacterium tumefaciens* into plant cells. (This system was used in the production of most of the genetically modified crop plants.) Interestingly, a very similar system is involved in the conjugational transfer of plasmid DNA from one bacterial cell to another.

Mycobacterial Cell Envelope

Although mycobacteria are not Gram-negative, their lipid-rich cell wall appears to have an overall structure similar to the Gram-negative cell wall. This was suggested by the observation that the hydrocarbon chains of the cell-wall lipids are in a state of very low fluidity and arranged in a direction perpendicular to the plane of cell surface (**Figure 13**). However, unlike in the outer membrane, in mycobacteria the least permeable portion of the cell wall appears to correspond to its inner part, where long, saturated hydrocarbon chains of mycolic acid residues are packed together. Indeed, this portion of the cell wall seems to have a very low fluidity, and very low permeability for lipophilic molecules is expected for the mycobacterial cell wall. Mycobacterial cell wall also has an exceptionally low permeability for hydrophilic solutes, even lower than the *P. aeruginosa* outer membrane in some species. Porins were identified in mycobacterial cell wall, although they are very different proteins from Gram-negative porins. The number and properties of these mycobacterial porins appear to explain the low hydrophilic permeability of the cell wall.

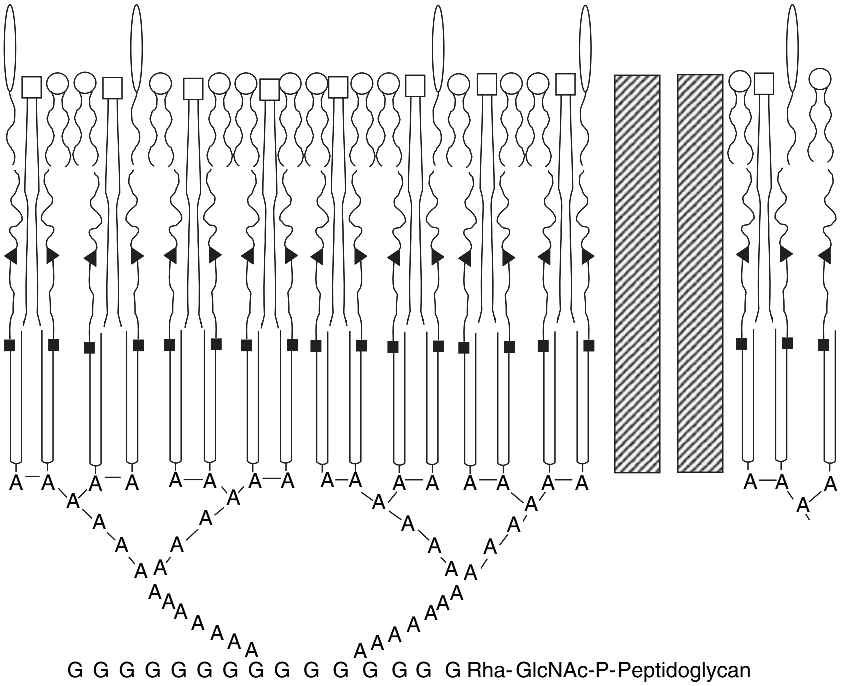

Figure 13 Model of mycobacterial cell wall. Arabinogalactan (A for arabinofuranose; G for galactofuranose) is linked covalently to peptidoglycan through a linker oligosaccharide rhamnosyl-*N*-acetylglucosamine-phosphate (Rha-GlcNAc-P). To the terminal arabinose residue is linked mycolic acid, a branched fatty acid with very long chains of uneven lengths (typically 24 and 60 carbons). Within the longer branch, there are a double bond or *cis*-cyclopropane group (solid squares) and a *cis* double bond, *cis*-cyclopropane group, or oxygen-containing group (solid triangles). The inner part of the envelope occupied by the most proximal part of the mycolic acid chains is expected to have a very low fluidity. The outer leaflet is assumed to contain lipids with short hydrocarbon chains (glycerophospholipids, glycopeptidolipids) and lipids with intermediate-length hydrocarbon chains (phenolic glycolipids, phthiocerol dimycocerosate). Porin is drawn as two apposed rectangles, although detailed crystallographic structures are known for some of them.

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Peptidoglycan (Murein)

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Polymerization of Peptidoglycan
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Glossary

cell envelope Is the set of layers that delimit and wrap a bacterial cell, normally composed of the cytoplasmic membrane and cell wall (sacculus) plus an outer membrane in Gram-negative bacteria.

muropeptide Each of the monomeric subunits of peptidoglycan.

peptidoglycan Is a polymer consisting of sugars and amino acids. Murein (from the latin *murus*, wall) is the particular form found in the bacterial cell walls; however, the generic term is most often used. Some Archea present structurally similar components named pseudomurein.

periplasm/periplasmic space Is the compartment between the cytoplasmic membrane and the outer membrane in Gram-negative bacteria.

sacculus Also known as the cell wall, is the rigid layer of the bacterial cell envelope made of peptidoglycan and juxtaposed to the external side of the cytoplasmic membrane.

turgor Is the osmotic pressure exerted by the cell contents against the cytoplasmic membrane as a consequence of the high intracellular concentration of metabolites and macromolecules.

Abbreviations

IWZ	inner wall zone
Mgt	monofunctional glycosyl-transferases
OM	outer membrane
OWZ	outer wall zone
PBP	penicillin-binding proteins
PGCT	peptidoglycan-derived cytotoxin
TCT	tracheal cytotoxin

Defining Statement

The peptidoglycan sacculus is a key structural component of the bacterial cell. Peptidoglycan metabolism is a complex process taking place in different cellular compartments. Present day knowledge on the characteristics and central metabolic pathways of peptidoglycan metabolism is presented. The dynamic character of cell wall biology is given particular consideration.

Introduction

Peptidoglycan is a fundamental component of the cell envelope of nearly all bacteria. It is a polymeric macromolecule made up of linear glycan chains cross-linked to each other by short peptide bridges. Peptidoglycan (or murein) is laid down on the outside of the cytoplasmic membrane as a covalently closed, net-like, macromolecular structure known as the cell wall or sacculus (small bag) (**Figure 1**). Preservation of cell integrity and determination of bacterial shape are the primary functions of the peptidoglycan. The net-like, covalently closed structure of the sacculus is particularly appropriate to maintain cell shape while standing the high (2 to >15 bar) turgor pressure exerted by the cytosol against the cytoplasmic membrane of the cell. Because the sacculus completely wraps the cell body, the cytoplasmic material has to adopt the shape of the relatively rigid sacculus (**Figure 2**). Therefore, variations in cell shape and size are constrained by a concurrent change of the sacculus itself. These characteristics together with the ability of isolated sacculi to accurately keep the shape of the original cell make the sacculus the bacterial equivalent to an exoskeleton (**Figure 2**). While bulk incorporation of new material is clearly necessary to enlarge the sacculus, the

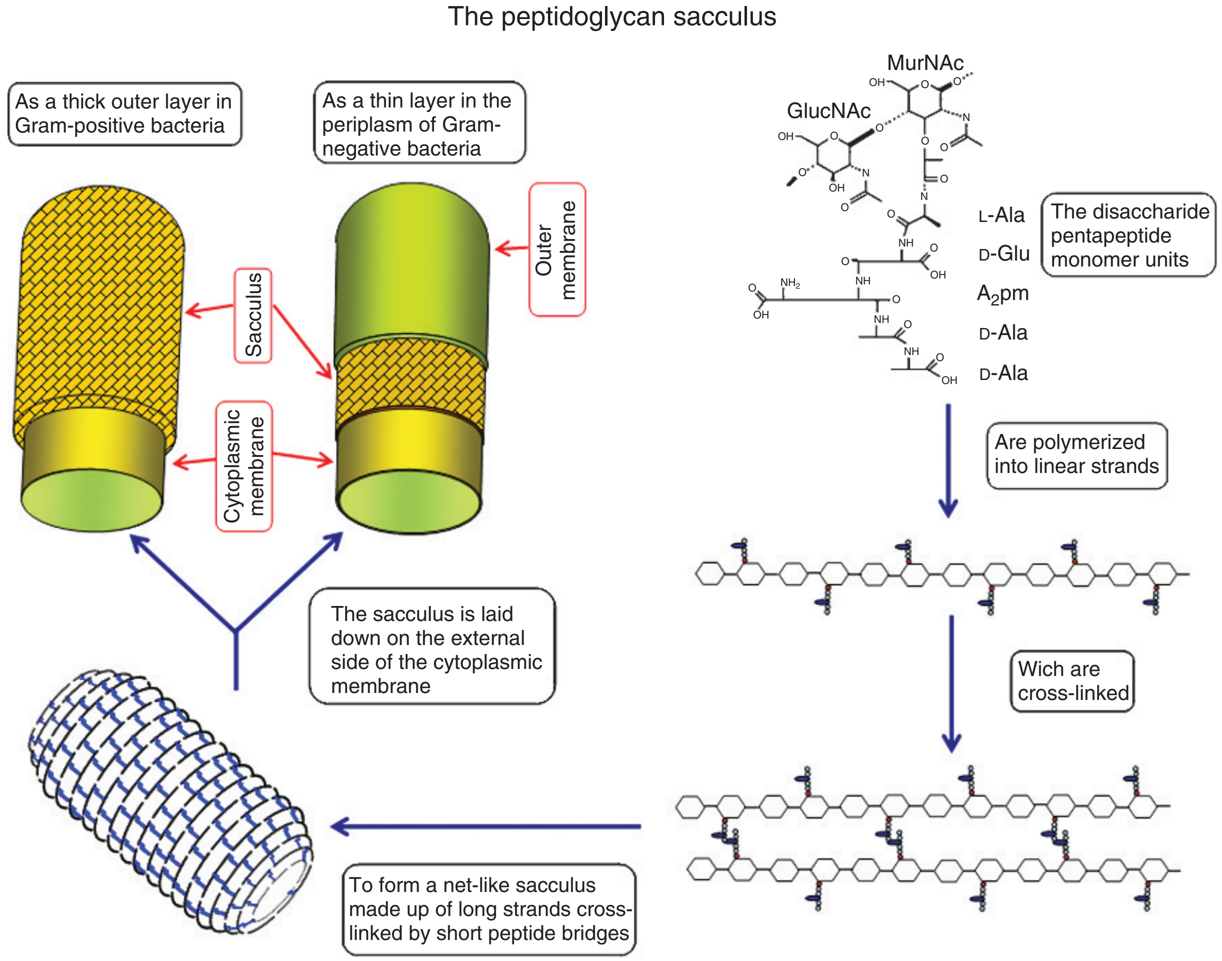
The peptidoglycan sacculus
As a thick outer layer in Gram-positive bacteria
As a thin layer in the periplasm of Gram-negative bacteria
Outer membrane
Sacculus
Cytoplasmic membrane
MurNAc
GlucNAc
L-Ala
D-Glu
A_2pm
D-Ala
D-Ala
The disaccharide pentapeptide monomer units
Are polymerized into linear strands
Wich are cross-linked
The sacculus is laid down on the external side of the cytoplasmic membrane
To form a net-like sacculus made up of long strands cross-linked by short peptide bridges

Figure 1 Basic structure and organization of the bacterial peptidoglycan sacculus.

spatial distribution and temporal regulation of insertion events define the final shape of the bacterium.

The sacculus serves further important roles as a scaffold to support the external layers of the cell envelope (S-layers and the outer membrane (OM) of Gram-negative bacteria); in the biogenesis of supramolecular structures (export complexes and flagella) and in protein export in Gram-positive bacteria. Moreover, peptidoglycan is critically involved in developmental processes as the generation of resistance forms (spores) and appendages of different function (prostheca).

Because peptidoglycan is exclusively found in bacteria and its biosynthetic pathway is both essential and highly conserved, it makes the ideal target for antibacterial drugs. Most of the commonly used antibiotics (β-lactams, glycopeptides, and phosphomycin) target enzymes involved in peptidoglycan biosynthesis.

A murein sacculus is present in all known bacteria with the remarkable exceptions of Mollicutes, Planctomycetes, Chlamydiae, and the scrub typhus causative agent *Orientia tsutsugamushi*. Interestingly, peptidoglycan-free microorganisms belong to largely unrelated phylogenetic branches, and colonize completely different habitats, from facultative parasites (*Mycoplasma*) to marine free living forms (*Planctomyces*). Although peptidoglycan has not been detected in Chlamydia, most of the biosynthetic genes are conserved, and Chlamydia are indeed sensitive to β-lactam antibiotics (the Chlamydial anomaly). This enigmatic phenomenon suggests the presence of some vestigial, but functionally important, form of peptidoglycan. The case of *O. tsutsugamushi* is also remarkable. This organism, formerly known as *Rickettsia tsutsugamushi*, has been separated from the genus Rickettsia mostly for the absence of a peptidoglycan sacculus, present in all other *Rickettsia*. Conversely, peptidoglycan layers entirely similar to bacterial sacculi are an essential structure in the photosynthetic organelles (cyanelles) of glaucocystophyte algae as *Cyanophora paradoxa*. This as well as the recent identification of peptidoglycan biosynthesis genes in green plants (five genes in *Arabidopsis thaliana*) and mosses (nine genes in *Physcomitrella patens*) constitutes strong

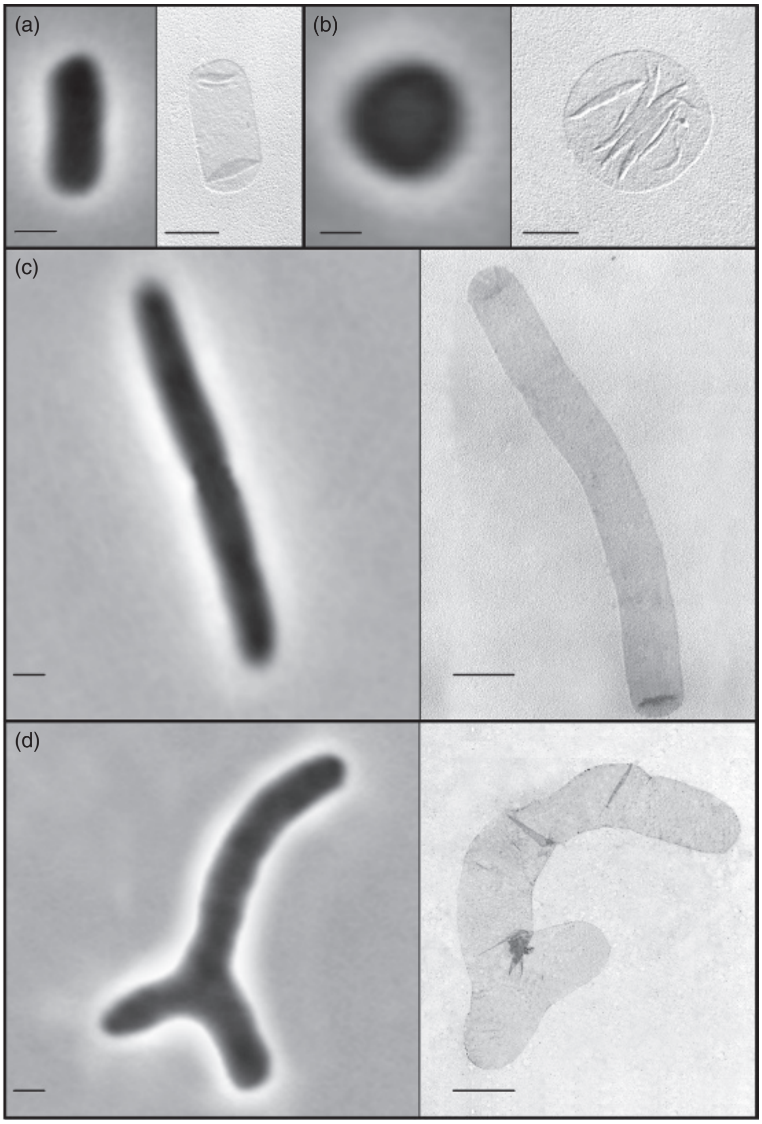

Figure 2 Peptidoglycan sacculi purified from shape mutants of *Escherichia coli*. Sacculi and whole cells of wild type and mutant strains affected in cell morphology are compared to show how purified sacculi retain shape of the original cell. (a) Wild-type *E. coli* with normal rod-like shape; (b) spherical shape mutant affected in the *rodA* gene; (c) filament-shaped cells of a division thermosensitive *ftsI* mutant; (d) branched shaped cells from a *dacA* mutant grown in the presence of the cell division inhibitor azthreonam. Left panels are phase-contrast images of intact cells, and right hand panels are TEM images of sacculi isolated from the corresponding strain. Images have been manipulated to approximate magnification of the phase-contrast and TEM images. The bars represent 1 μm. Notice the thinness of sacculi.

evidence supporting the endosymbiotic origin of eukaryotic organelles.

The exclusivity of peptidoglycan as a bacterial cell surface component has been successfully exploited by higher eukaryotes (plants and animals) as a means to recognize and fight, bacterial infections. Indeed, peptidoglycan is one of the main targets for 'pattern recognition proteins', as the Toll family, which are at the basis of the innate immune response.

Because of peptidoglycan extracellular location and covalently closed nature, its biosynthesis represents a specially challenging problem to the bacterial cell. Precursors are synthesized in the cytoplasm but polymerization and attachment to the preexisting sacculus takes place in the external environment, where there is no readily available source of metabolic energy. Besides, insertion of new precursors is not enough by itself to promote enlargement of the preexisting structure. Concomitant cleavage of chemical bonds in the sacculus is a strict requirement for growth. Therefore, both biosynthetic and degrading enzymes must work in concert for the sacculus to grow harmonically. In addition to the main biosynthetic pathway, rather complex by itself, peptidoglycan metabolism is further complicated by the nested intervention of pathways responsible for maturation, adaptive modifications, and recycling, not to mention species-specific pathways for the generation of resistance forms and/or specific appendages.

I will focus here on the more universal aspects of peptidoglycan composition, biosynthesis, and physical properties. Metabolism of the sacculus is intrinsically coupled with virtually all aspects of bacterial cell growth, division, development, and morphogenesis. However, treatment of such aspects in any detail would require a much more extensive and intensive treatment, out of the scope of this presentation.

Peptidoglycan Chemical Composition

Peptidoglycan composition and biosynthesis are fairly well established for a few model systems, but the base of knowledge is still relatively small. Not more than about 150 species have been studied in any detail and unexpected findings may indeed happen.

Traditionally, bacteria are divided into two main groups according to their response to the Gram stain: Gram-positive and Gram-negative. The distinction was established well before discovery of peptidogycan, but reflects the basic dichotomy in the organization of the bacterial cell envelope. Gram-negative bacteria are characterized in structural terms by the presence of a thin peptidoglycan layer, surrounded by an asymmetric OM made of an outer lipopolysaccharide leaflet and an inner phospholipid leaflet. The OM constitutes a permeability barrier to molecules of more than a few hundred Dalton (a typical cutoff value is around 600 Da). As a result, the peptidoglycan layer is confined in a particular compartment defined by the OM and the cytoplasmic membrane; the periplasmic space (**Figure 1**). Conversely, Gram-positive bacteria display thick peptidoglycan layers, are devoid of OM, and have accessory polymers linked to the cell wall peptidoglycan (**Figure 1**). Gram-positive bacteria show a remarkable degree of chemical and structural variability in their cell walls, in contrast with the rather homogenous nature of Gram-negative bacteria on these respects.

Peptidoglycan Basic Structure

The basic structural features of peptidoglycan are polymeric glycan strands cross-linked by short peptides. The canonical monomeric unit consists of the disaccharide *N*-acetylglucosamine (GlcNAc) (β-1 $\rightarrow$ 4) *N*-acetylmuramic acid (MurNAc) substituted at the D-lactoyl group of MurNAc by a peptide stem with the sequence L-Ala-(γ)-D-Glu-*meso*A_2pm-D-Ala-D-Ala, known in short as the disaccharide pentapeptide. Successive monomers are linked to each other by (β-1 $\rightarrow$ 4) glycosidic bonds to form the glycan strands. Glycan strands become in turn cross-linked to each other through the stem peptides, normally between the amino acid at position 4 (invariably a D-Ala) in one strand and the dibasic amino acid at position 3 of the neighboring strand (**Figure 1**). Cross-linking can connect both amino acids either directly or through an intermediate short peptide (interpeptide bridge) that is added to the dibasic amino acid in the course of monomer unit biosynthesis. The monomeric subunits of peptidoglycan are commonly known as muropeptides.

The monomer is an unusual biomolecule characterized by the following features: (1) the presence of the rare MurNAc molecule, exclusively found in the bacterial sacculus and, oddly enough, in tissues of some gastropods; (2) the presence of a (γ)-bonded D-Glu residue; (3) the occurrence of L-D and D-D peptide bonds, never found in proteins; (4) the existence of nonprotein amino acids (*meso*A_2pm, L-ornithine, L-lanthionine); and (5) presence of a C-terminal D-Ala-D-Ala dipeptide. These basic features are virtually universal. However, peptidoglycan from individual species normally presents modifications on this common theme, both in the glycan and in the peptide moieties.

Structural Parameters of Peptidoglycan

The parameters more often used to describe the structural properties of peptidoglycan sacculi are cross-linkage and average glycan strand (chain) length.

Cross-linkage reflects the proportion of muropeptides, which are covalently linked through peptide bonds bridging the respective stem peptides. Cross-linkage is normally expressed as the molar fraction of cross-linked with respect to total muropeptides in percentage. The higher the cross-linkage, the stronger, and stiffer, the peptidoglycan. Reported values show a large variability from species to species, and also in response to environmental conditions (see 'Postinsertional metabolism of peptidoglycan'). Among Gram-positives, cross-linkage is habitually high and values close to the theoretical 100% maximum have been reported (93% in *Staphylococcus aureus*). However, values for Gram-negative bacteria are much lower, ranging from 20 to 40% in most cases. This large difference is a consequence of the mostly monolayered arrangement of sacculi from Gram-negative bacteria. Geometrical constrains limit the maximum theoretical cross-linkage to about 50% for a monolayer.

The length of the glycan strands is also a key structural parameter to understand the properties of the sacculus. Experimental determination of this parameter has been possible only in a very small number of cases. A major limitation comes from the fact that the length of glycan strands is not uniform but rather follows a very wide distribution. Precise data on the distribution of glycan strand lengths have only been obtained for *Escherichia coli*. In this organism, the average length is about 30 monomers, with a modal value of only 10 monomers. The distribution is very wide and strongly skewed toward the shorter length classes; nevertheless about 30% of total muropeptides are in relatively long strands of more than 30 monomer units. In other bacteria only an average value could be determined at best. The few data available indicate that peptidoglycan is predominantly made up of short glycan strands, with average lengths from 20 to 50 monomer units (*Helicobacter pylori*, *Bacillus subtilis*, *Bacillus licheniformis*, *Streptococcus faecium*). However, as found for *E. coli*, glycan strands considerably longer than average might contribute substantially to the sacculus structure. It is important to realize that because a monomer unit is about 1 nm in length, and most bacteria are about or greater than 1 μm in circumference, single glycan chains are by far too short to loop around the cell; therefore a number of glycan strands have to be arranged 'head to tail' to encircle the cell.

Variations in the Glycan Moiety

The glycan backbone, which should consist exclusively of poly(GlcNAc-MurNAc), becomes invariably modified. Modification of the glycan moiety occurs in general at the late stages of synthesis, either associated to polymerization of the glycan strands, or after insertion of new strands into the sacculus. The glycan strands of Gram-negative species have a residue of $(1 \rightarrow 6)$anhydro MurNAc as the 1-terminal saccharide, eliminating the reducing character which the peptidoglycan should otherwise have. In Gram-positive bacteria, covalent attachment of additional cell wall polymers, as teichoic acids, through phosphodiester bonds to GlcNAc or MurNAc is virtually universal.

Many species present secondary modifications in the glycan strands often conferring resistance against degrading enzymes like the widespread muramidases (lysozyme). O-acetylation, N-deacetylation, and N-glycolylation are the more common secondary modifications of glycan strands.

The most frequent modification of glycan chains is O-acetylation. This reaction, first detected in *Micrococcus luteus*, consists of the addition of an acetyl group to the C_6-OH of MurNAc residues, to form 2,6-*N*,*O*-diacetyl-MurNAc. O-acetylation occurs both in Gram-positive and in Gram-negative species, including many important pathogens (*S. aureus*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *H. pylori*, etc.). O-acetylation occurs at the level of polymerized peptidoglycan by means of *O*-acetyltransferases. Two types of *O*-acetyltransferases have been described: OatA-type are single integral membrane proteins that simultaneously perform transport of acetate through the cytoplasmic membrane and transfer onto the MurNAc residues; Pat-type are composed of two proteins, one for acetate transport and the second for transfer to MurNAc. O-acetylation renders peptidoglycan resistant to most known muramidases and contributes to virulence of pathogens as Staphylococci. Besides, O-acetylation prevents lysozyme clearing of cell wall fragments from blood serum after bacterial infection, and contributes to induction of rheumatoid arthritis in humans.

Elimination of the N-acetyl groups at position 2 of either amino sugar increased peptidoglycan resistance to lysozyme in several important pathogens such as *Bacillus anthracis*, *Listeria monocytogenes*, and *S. pneumoniae*. In all cases known, N-deacetylation takes place on polymerized peptidoglycan, and known deacetylases are predicted to be extracytoplasmic enzymes.

The presence of glycolyl residues instead of acetate at the amino group of MurNAc was first found in *Mycobacterium smegmatis*. Later it was clearly stated that N-glycolylation was a hallmark of the Actinomycetales. Contrary to the modifications discussed above, N-glycolylation occurs at the stage of UDP-linked precursors in the biosynthesis of the monomeric subunit by the action of a mono-oxygenase in the presence of molecular oxygen and NADPH. As in the previous cases, impairment of the glycolylating enzyme results in hypersensitivity against lysozyme and β-lactam antibiotics.

Variations in the Sequence of the Stem Peptide

The stem peptide sequence shows a considerable degree of variability but, in most cases alterations can be viewed as conservative, inasmuch as the basic features are concerned. Variations in the stem peptide sequence are dictated either by the specificity of the biosynthetic enzymes, or by the postsynthetic modification of the otherwise standard sequence.

Replacement of the canonical amino acid (L-Ala) at position 1 is rare and only a few cases are known where Gly (*Mycobacterum leprae*, *Brevibacterium imperiale*) or L-Ser (*Butyribacterium rettgeri*) substitute for the regular L-Ala. In all species studied so far, the amino acid at position 2 is invariably D-Glu.The third amino acid in the stem, usually a dibasic amino acid, shows the higher variability. Most often, A_2pm (most Gram-negative bacteria, but also Bacilli and Mycobacteria) or L-Lys (most Gram-positive species) occupy the third position. However, in a number of species this position is occupied by unusual dibasic amino acids as L,L-diaminopimelic acid (*Streptomyces albus*) L-ornithine (Spirochetes, *Thermus thermophilus*, *Deinococcus radiodurans*), *meso*-lanthionine (*Fusobacterium*), or L-2,4,diamino butyrate (*Corynebacterium aquaticum*). In a few instances, mono amino acids also take the third position as in *Corynebacterium poinsettiae* (L-homoserine) and *Erysipelothrix rhusiopathiae* (L-Ala). Another interesting exception is the case of a few species where the third position is occupied by two amino acids as in *Bifidobacterium globosum* in which either L-Lys or D-Lys can be at position 3 because of poor substrate specificity of the adding enzyme.

Amino acids at positions 4 and 5 consist almost invariably of D-Ala. The D-Ala-D-Ala terminal dipeptide is perhaps the most widely known peptidoglycan hallmark. However, a certain proportion of Gly is often found at these positions because of lack of selectivity of D-Ala-D-Ala ligase, the enzyme making the dipeptide. The proportion of Gly is normally quite low ($\leq$1%) but in some species, such as *Caulobacter crescentus,* can be as high as 19%. Undoubtedly, the most relevant alteration known has been recently identified in some strains of Enterococci. In such strains, D-lactate or D-Ser substitute for the D-Ala at position 5. The modification is associated with development of high level resistance against the antibiotic vancomycin. Vancomycin binds D-Ala-D-Ala with high affinity and blocks the late steps of peptidoglycan biosynthesis. However, the depsipeptide D-Ala-D-Lac is a very poor substrate for vancomycin and therefore substitution of D-Lac for D-Ala prevents antibiotic action. Actually, this mechanism of resistance is a serious clinical problem.

Secondary Modifications of the Stem Peptide

Other variations in the chemical composition of monomer peptide stem (amidation, acetylation, attachment of additional amino acids, etc.) occur at later stages by modification of the otherwise regular monomeric subunit. These modifications more often affect amino acids at positions 2 and 3. Amidation of D-Glu and *meso*-A_2pm at the α- and ε-carboxyl groups, respectively, is quite common in Gram-positive bacteria. The membrane-bound form of the complete monomer is the substrate for amidation. Hydroxylation of D-Glu, *meso*-A_2pm, and L-Lys has been found is some species in direct relation with oxygenation levels. Addition of aminated compounds to the α-carboxyl group of D-Glu at position 2 has been documented in some organisms, as *M. luteus* (Gly), *Arthrobacter* (Glycine amide), and also in peptidoglycan from *C. paradoxa* cyanelles (*N*-acetyl putrescine). In many Gram-positive species the third amino acid is often modified at the level of Lipid I by addition of a short (1-7 amino acids), highly variable peptide, to the free amino group that acts as an intermediate bridge in cross-linking reactions between peptide stems.

The peptide stem constitutes in addition the anchoring point for specific cell envelope proteins. Gram-negative species often present a small size lipoprotein (the 58-amino-acid Braun's lipoprotein in *E. coli*) as the only known molecule covalently bound to murein. Murein-bound lipoprotein anchors the OM to the sacculus. The acylated N-terminal domain of the lipoprotein is buried into the phospholipid inner leaflet of the OM, while the ε-amino group of the C-terminal L-Lys becomes attached to the α-carboxyl group of *meso*-A_2pm by means of a transpeptidation reaction, effectively bridging both cell envelope layers.

Gram-positive bacteria in general present a larger number of peptidoglycan-bound proteins, often involved in pathogenic processes. Binding is mediated by cytoplasmic membrane proteins denominated sortases. Sortases recognize a specific sequence in the protein and transfer the bulk of the protein to the side chain amino group of the dibasic-amino acid at position 3 through a transpeptidation reaction. Binding of proteins by sortase seems to take place at the Lipid II level (see 'Synthesis of lipid intermediates'), in Gram-positive bacteria as exemplified by sortase A from *S. aureus*.

Biosynthesis of Peptidoglycan Monomer Units

Biosynthesis of peptidogylcan has been the matter of active research for more than 60 years. From these efforts an overall view of the process thought to be valid for all bacteria has emerged.

Peptidoglycan biosynthesis is traditionally divided into two stages on the basis of the cell compartments where it takes place. The first stage concerns assembly of the monomer unit in the intracellular space by enzymes present in the cytoplasm or in the cytoplasmic membrane inner leaflet. The end product of the first stage is a membrane-anchored, lipid intermediate: disaccharide-pentapeptide pyrophosphate undecaprenol (Lipid II) (**Figure 3**). The second stage addresses the extracellular polymerization steps that take place on the outer side of the cytoplasmic membrane and use the lipid intermediate as the initial substrate (**Figure 4**). Concomitant with polymerization, nascent peptidoglycan is inserted into the macromolecular structure of the sacculus, in a way promoting further physical expansion of the preexisting structure and ensuring preservation of proper shape.

Assembly of the monomer can be subdivided into four stages: synthesis of UDP-GlcNAc; synthesis of UDP-MurNAc; sequential formation of UDP-MurNAc-peptides; and synthesis of the lipid-linked intermediates (**Figure 3**).

Synthesis of UDP-GlcNAc

Synthesis of UDP-GlcNAc occurs in four reactions. The first reaction is the conversion of fructose-6-phosphate and glutamine into glucosamine-6-phosphate by the GlmS protein (glucosamine-6-phosphate-synthase), which is isomerized to glucosamine-1-phosphate by GlmM protein (phosphoglucosamine mutase). Activity of GlmM is regulated by phosphorylation of a serine at the active site, and seems to be the checkpoint in the regulation

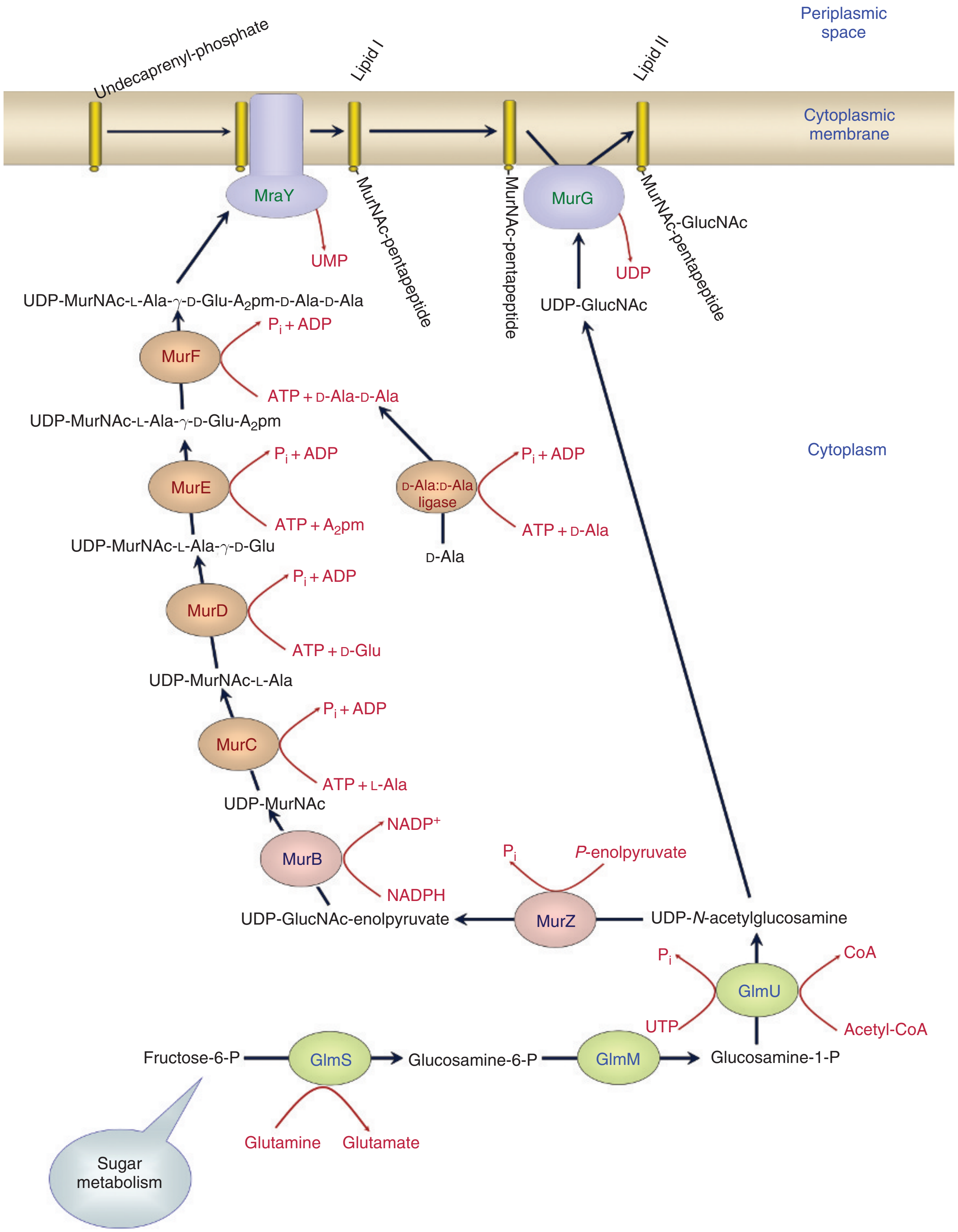
Periplasmic space
Undecaprenyl-phosphate
Lipid I
Lipid II
Cytoplasmic membrane
MraY
UMP
MurNAc-pentapeptide
MurNAc-pentapeptide
MurG
UDP
-GlucNAc
MurNAc-pentapeptide
UDP-GlucNAc
UDP-MurNAc-L-Ala-γ-D-Glu-A$_2$pm-D-Ala-D-Ala
MurF
P$_i$ + ADP
ATP + D-Ala-D-Ala
UDP-MurNAc-L-Ala-γ-D-Glu-A$_2$pm
MurE
P$_i$ + ADP
ATP + A$_2$pm
D-Ala:D-Ala ligase
P$_i$ + ADP
ATP + D-Ala
D-Ala
Cytoplasm
UDP-MurNAc-L-Ala-γ-D-Glu
MurD
P$_i$ + ADP
ATP + D-Glu
UDP-MurNAc-L-Ala
MurC
P$_i$ + ADP
ATP + L-Ala
UDP-MurNAc
MurB
NADP$^+$
NADPH
P$_i$
P-enolpyruvate
UDP-GlucNAc-enolpyruvate
MurZ
UDP-N-acetylglucosamine
P$_i$
GlmU
CoA
UTP
Acetyl-CoA
Fructose-6-P
GlmS
Glucosamine-6-P
GlmM
Glucosamine-1-P
Glutamine
Glutamate
Sugar metabolism

Figure 3 Schematic representation of peptidoglycan monomer unit biosynthesis. The individual stages, synthesis of *N*-acetylglucosamine, synthesis of *N*-acetylmuramic acid, addition of the stem peptide, and synthesis of lipid-linked precursors, are differentiated by colors. Cosubstrates required for each step are highlighted in red. Enzymes are indicated by their short names derived from the genetic *E. coli* nomenclature, which correspond to: GlmS, glucosamine-6 phosphate-synthase; GlmM, phosphoglucosamine mutase; GlmU, glucosamine-1-phosphate acetyltransferase plus *N*-acetyl-glucosamine-1-phosphate uridyltransferase; MurA, UDP-*N*-acetylglucosamine enolpiruvyl transferase; MurB, UDP-*N*-acetyl-enolpiruvylglucosamine reductase; MurC, UDP-*N*-acetylmuramic acid:L-alanine ligase; MurD, UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase; MurE, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase; MurF, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase; MurG, UDP-*N*-acetylglucosamine: *N*-acetylmuramyl(pentapeptide)-*P*-*P*-undecaprenol-*N*-acetylglucosamine transferase; and MraY, phospho-*N*-acetylmuramyl-pentapeptide translocase.

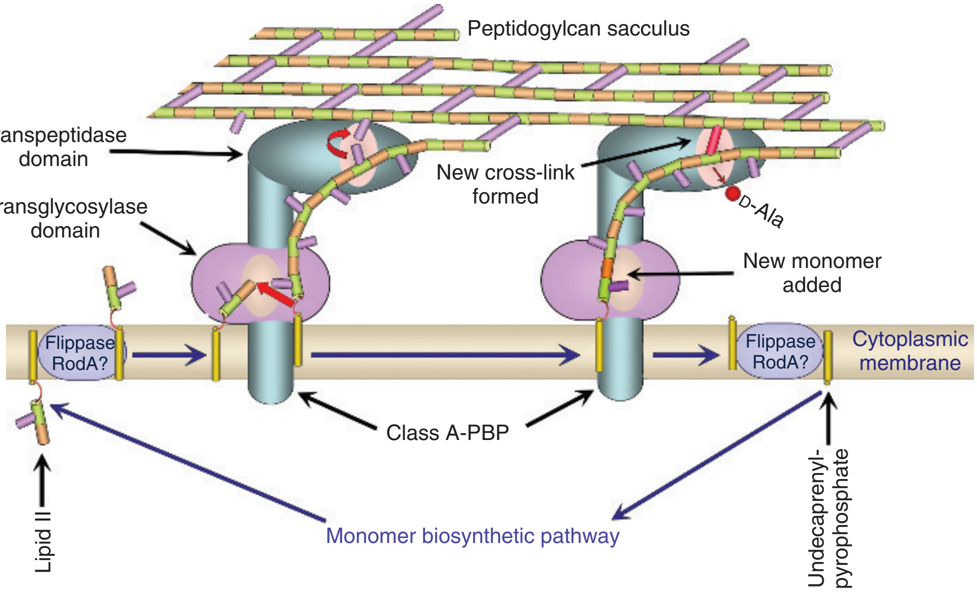

Figure 4 Schematic representation of peptidoglycan polymerization. In a first step, Lipid II precursor molecules must be transferred to the external side of the cytoplamic membrane. No flippase has been actually demonstrated but RodA (and the similar protein FtsW) is a likely candidate. Once on the outside, a Lipid II molecule interacts with the transglycosylase domain of a bifunctional class-A high-molecular-weight PBP. A nascent glycan strand, already attached to the sacculus at the distal end, is transferred from undecaprenyl-phosphate onto the C_4-OH of the Lipid II molecule in the transglycosylase domain, causing the glycan strand to grow by one unit. A molecule of undecaprenyl-pyrophophate is also released and upon transfer to the cytoplasmic side of the membrane, undergoes a new round of synthesis. Concomitantly, a stem peptide from the nascent chain and a second stem peptide in the preexisting sacculus find the right relative positioning at the transpeptidase domain active site and become cross-linked by transfer of the peptide bond between the two terminal D-Ala residues of the stem peptide in the nascent glycan strand to the dibasic amino acid in the stem-peptide at the sacculus, releasing a molecule of D-Ala. Iteration of the process leads to coordinated glycan strand linear polymerization and insertion of the nascent strands into the net-like structure of the sacculus.

of UDP-GlcNAc synthesis. The final two steps are catalyzed sequentially by the bifunctional enzyme GlmU. The C-terminal domain of GlmU has glucosamine-1-phosphate acetyltransferase activity and catalyzes transfer of an acetyl group from acetyl-CoA to the amino group of glucosamine-1-phosphate, while the N-terminal domain, endowed with *N*-acetyl-glucosamine-1-phosphate uridyltransferase activity, uridylates the product of the previous reaction in the presence of UTP, producing UDP-GlcNAc. The intracellular concentration of UDP-GlcNAc is the limiting factor for the subsequent steps in peptidoglycan biosynthesis.

Synthesis of UDP-MurNAc

UDP-MurNAc is synthesized from UDP-GlcNAc in a two-step process: first transfer of enoylpyruvate from phosphoenolpyruvate to the C_3-OH of a UDP-GlcNAc molecule by the MurA transferase (UDP-*N*-acetylglucosamine enolpyruvyl transferase) to yield UDP-GlcNAc-enolpyruvate and second reduction of enolpyruvate to D-lactate by the MurB reductase (UDP-*N*-acetyl-enolpyruvylglucosamine reductase) in the presence of NADPH to make UDP-MurNAc.

Addition of the Stem Peptide

Addition of the stem peptide is a sequential process in which L-Ala, D-Glu, a dibasic amino acid, most often *meso* A_2pm or L-Lys, and the dipeptide D-Ala-D-Ala are incorporated into UDP-MurNAc to produce UDP-MurNAc-pentapeptide. Amino acids are added stepwise by the highly specific synthases MurC (UDP-*N*-acetylmuramic acid:L-alanine ligase); MurD (UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase); MurE (UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate:*meso*-diaminopimelate ligase); MurF (UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-diaminopimelate:D-alanyl-D-alanine ligase); and D-alanine:D-alanine ligase, which synthesizes the dipeptide D-Ala-D-Ala the substrate for MurF. All these ligases are ATP-dependent, and have a similar catalytic mechanism: activation of the C-terminal amino acid of the growing nucleotide precursor to an acyl-phosphate intermediate at the expense of ATP, followed by a nucleophilic attack by the NH_2 group of the added amino acid leading to release of phosphate and formation of a new peptide bond. The specificity of both MurE and MurF is variable according to the dibasic amino acid present at position 3 of the stem peptide, a species-specific feature.

Synthesis of Lipid Intermediates

Attachment of the soluble precursors to the isoprenoid lipid undecaprenyl phosphate (bactoprenol) in the cytoplasmic membrane marks the beginning of the membrane-bound phase of peptidoglycan biosynthesis. The first reaction consists in the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to the membrane acceptor undecaprenyl-phosphate to yield MurNAc(pentapeptide)-pyrophosphoryl-undecaprenol (Lipid I) and UMP. The reaction is catalyzed by the MraY translocase (phospho-*N*-acetylmuramyl-pentapeptide translocase), an integral membrane protein. In a consecutive reaction, the MurG transferase (UDP-*N*-acetylglucosamine:*N*-acetylmuramyl (pentapeptide)-*P*-*P*-undecaprenol-*N*-acetylglucosamine transferase) catalyzes the addition of a residue of GlcNAc from UDP-GlcNAc to the MurNAc moiety of a Lipid I molecule through a (β-1 → 4) glycosidic bond giving the completed lipid linked monomer unit UDP-GlcNAc-(β-1 → 4)-MurNAc(pentapeptide)-pyrophosphoryl-undecaprenol (Lipid II). In *E. coli* MurG is a peripheral membrane protein associated to the inner leaflet of the cytoplasmic membrane.

In growing cells the proportion Lipid I to UDP-MurNAc-pentapeptide is very low, in the 1% range, indicating that availability of undecaprenyl phosphate is a limiting factor for the membrane-bound steps of peptidoglycan biosynthesis. The scarcity of Lipid I and Lipid II (ca. 700 and 2000 molecules per cell in *E. coli*) implies a high turnover rate (about 1 s). This in turn constrains free diffusion of lipid precursors as an efficient way to sustain ongoing synthesis, and supports the existence of associations with the transferases and later acting proteins in peptidoglycan synthesizing complexes. Indeed, recent evidence gathered from *E. coli* supports an association of MurG with the synthetic complexes responsible for longitudinal growth of the sacculus.

Addition of Interpeptide Bridges

In most Gram-positive bacteria cross-linking between peptidoglycan stem peptides occurs with the concourse of a short peptide, which is added to the dibasic amino acid at position 3. The size and composition of the interpeptide bridges are species-specific features. Size ranges from 1 to 7 amino acids and composition include Gly as well as D-(D-Ser, D-Glu, D-Asx) and L-(L-Ala, L-Glu) amino acids. The nature of the interpeptide bridges is the main cause of variability in peptidoglycan. However, knowledge of the enzymes involved (branching enzymes) is still limited. Synthesis of the interpeptide bridge occurs by the sequential addition of amino acids to a pentapeptide precursor. The precursor substrate for branching enzymes is variable amongst species, for instance, in *S. aureus* it is Lipid II, but in other species it can be Lipid I and also UDP-MurNAc-pentapeptide. Furthermore, in some instances the branching enzymes use more than one substrate (Lipid II and UDP-MurNAc-pentapeptide in *Enterococcus faecalis*). Two different mechanisms coexist in dependence of the nature of the amino acid to be added. L-amino acids as well as Gly are activated as amino acyl-tRNAs and added to the precursor by the nonribosomal peptide-forming enzymes Fem transferases. D-amino acids are incorporated by enzymes of the ATP-grasp family. These enzymes activate the amino acids as acyl-phosphates at the expense of ATP, and catalyze ligation of the activated carboxyl to different chemical groups, in particular amino and imino nitrogens.

Polymerization of Peptidoglycan

The second stage in peptidoglycan synthesis is polymerization of monomer units and incorporation of the new polymeric product into the preexisting sacculus in such a fashion as to promote growth and preserve the correct cell shape.

The polymerization stages are performed by two different membrane-bound activities: glycosyl transferases that catalyze formation of linear glycan polymers and peptidyl transferases (transpeptidases) that catalyze cross-linking of stem peptides. In growing bacteria formation of glycan strands (tranglycosylation) and cross-linking of stem peptides (transpeptidation) are continuous, tightly coupled reactions. However, detailed analysis of the process indicates that polymerization of the monomer proceeds by transglycosylation and precedes cross-linking of the nascent glycan strands by transpeptidation. It is important to realize that transpeptidation is not only responsible for the cross-linking of nascent material, but also for its incorporation into the sacculus (**Figure 4**).

Translocation of Lipid-Linked Precursors

Polymerization of peptidoglycan is an extracellular process, but the immediate precursor Lipid II is synthesized in the intracellular side of the cytoplasmic membrane. Therefore, the Lipid II units must be translocated to the external side of the membrane (**Figure 4**). In spite of its relevance, lipid-precursor translocation remains one of the least known stages of peptidoglycan synthesis. Translocation of the scarce and highly hydrophilic Lipid II at a rate fast enough to keep apace with polymerization almost certainly requires the catalytic intervention of specialized proteins (flippases). However, no Lipid II flippase has been unambiguously identified up to now. In the model organism *E. coli*, the proteins RodA and FtsW have been proposed as putative flippases. These proteins are required by the essential peptidoglycan syntheses penicillin-binding proteins (PBP) 2 and 3 (see below) respectively, to fulfill their functions. RodA and

FtsW are similar integral membrane proteins, with ten membrane-spanning regions, and had been proposed to work as tunneling devices to channel and provide Lipid II to their cognate PBPs. Paralogues to *E. coli rodA* and *ftsW* have been found in other bacterial species, both Gram-positive and Gram-negative.

Penicillin-Binding Proteins; the Key Peptidoglycan Synthases

PBPs are a set of minor cytoplasmic membrane proteins ubiquitous in bacteria. PBPs are the specific targets for β-lactam antibiotics and are critically involved in the late stages of peptidoglycan synthesis. PBPs are defined by their ability to covalently bind β-lactams yielding stable, enzymatically inactive, acyl-enzyme complexes. This property facilitated identification of PBPs by specifically labeling with radioactive or fluorescent β-lactams. PBPs apparently share a common topology; they are anchored to the cytoplasmic membrane through a transmembrane domain, but the bulk of the protein is exposed to the external environment in close apposition to the sacculus, the PBPs insoluble substrate. Virtually, every known bacteria possesses a set of PBPs, but number, size, abundance, and β-lactam-binding spectrum are variable from species to species.

PBPs are habitually classified as high- (>45 kDa) and low- (<45 kDa) molecular-weight PBPs (HMW- and LMW-PBPs respectively). The difference in molecular weight does indeed reflect a functional dichotomy: while HMW-PBPs have polymerase activity, LMW-PBPs are more often involved in cleavage of peptidoglycan peptide bonds and display endopeptidase and carboxypeptidase activities. The number of reactions that a single polypeptide is able to catalyze differentiates two classes of HMW-PBPs: the class A HMW-PBPs, bifunctional enzymes able to catalyze both polymerization of the glycan strands (transglycosylation) and cross-linking of the stem peptides (transpeptidation), and class B HMW-PBPs that are monofuctional enzymes endowed with transpeptidase activity only.

Genetic analysis of HMW-PBPs in known bacterial genomes embodying more than 400 class A, and 300 class B HMW-PBPs demonstrated a clear modular architecture. Both classes present a short cytoplasmic N-terminal segment, followed by a single transmembrane domain followed in class A enzymes by the transglycosylase module, and in class B by a shorter module of unknown function, followed in turn by the C-terminal transpeptidase (peptidyl transferase) module responsible for cross-linking and β-lactam binding. The intermediate module in class B enzymes has been proposed to be involved in interactions with other membrane proteins. LMW-PBPs are monofunctional peptidyl transferases most often endowed with D,D-carboxypeptidase activity and with D,D-endopeptidase activity. As above, the active site of the peptidyl transferase is the target for β-lactam binding. Proteins of this group have an unusual architecture; the peptidyl transferase domain is encoded after a cleavable peptide signal and the protein is anchored to the membrane through a *C*-terminal transmembrane domain or amphipathic helix.

Synthesis of Linear Peptidoglycan Polymers

Polymerization of peptidoglycan Lipid II units is catalyzed by the bifunctional transglycosylase-transpeptidase class A HMW-PBPs. Many class A HMW-PBPs are known, but most of present day knowledge derives from *E. coli* PBP1B, the paradigmatic peptidoglycan synthase.

The full-length enzymatically active PBP1B seems to be a dimer. The most widely accepted mechanism for peptidoglycan polymerization involves the repetitive addition of disaccharide-pentapeptide units from Lipid II at the reducing end of a growing glycan chain attached to a second molecule of undecaprenyl-pyrophosphate. In such a scheme, the undecraprenyl-pyrophosphate-bound growing glycan chain acts as the glycosyl donor, and is transferred to the C_4-OH of the GlcNAc molecule in a Lipid II molecule that becomes the glycosyl acceptor substrate. While the nascent glycan strand grows by a monomer unit each cycle, the reaction generates free undecaprenol-pyrophosphate, which is transferred back to the cytoplasmic side of the membrane, hydrolyzed to undecaprenol phosphate, and reused for a new addition cycle (**Figure 4**).

The exact catalytic mechanism for transglycosylation is still uncertain. It is likely that the tranglycosylase domain of the PBP recognizes the sugar moieties of both the Lipid II and the terminal section of the growing glycan chain, as is characteristic for other related glycosyl transferases. It has been proposed that the γ-COOH of an active-site Glu residue (Glu_{223} in *E. coli* PBP1B) donates its proton to the phosphoester bond of the growing chain to give an oxicarbenium cation, which then undergoes nucleophilic attack by the 4-OH of the Gluc-NAC in the Lipid II molecule. Additional Asp and Glu residues in the active site (Asp_{234} and Glu_{290} in *E. coli* PBP1B) are likely involved in the stabilization of the oxicarbenium and activation of the C_4-OH group.

As mature peptidoglycan is devoid of any linkage to undecaprenyl phosphate, some enzymatic reaction must release nascent polymers from the membrane-anchoring undecaprenyl-phosphate moiety. Such enzyme(s) could play an important role in determining the length of the resulting peptidoglycan strands. However, there is no knowledge for any such enzyme so far. Release of the nascent glycan strand from undecaprenol-pyrophosphate in Gram-negative bacteria results in the generation of (1 → 6) anhydro MurNAc, which becomes a tag for the glycan chain terminal muropeptide. Formation of (1 → 6) anhydro MurNAc is thought to occur because transglycosylases may enter an idling state that results in an intramolecular glycosyl transfer of the nascent glycan strand onto the C_6-OH of the MurNAc residue, instead of the C_4-OH of a nearby Lipid II molecule. A possible alternative is that glycan strands are polymerized in a continuous fashion, and then are trimmed by the so-called lytic-transglycosylases enzymes able to split glycan polymers generating (1 → 6) anhydro MurNAc (see 'Brake to make; peptidoglycan hydrolases'). Presence of (1 → 6) anhydro MurNAc in the glycan strand terminal muropeptides is the reason for the nonreducing character of peptidoglycan in Gram-negative bacteria.

Class A HMW-PBPs clearly take the burden of Lipid II polymerization. Nevertheless, membrane-bound monofunctional glycosyl-transferases (Mgt), able to polymerize Lipid II, have been identified and biochemically characterized in a number of bacteria (*M. luteus*, *E. coli*, *S. pneumoniae*). Indeed, Mgts from these species often account for most of the Lipid II glycosyl transferase activity in '*in vitro*' assays with cell extracts. Further genomic analyses lead to the identification of putative Mgts in a much larger set of unrelated bacteria. Mgts share considerable similarity with the glycosyl transfer domains of class A PBPs.

An intriguing aspect of peptidoglycan synthetic transglycosylases, indeed of most enzymes acting on macromolecular peptidoglycan, is their high redundancy. For instance, both *E. coli* and *S. pneumoniae* have at least four different enzymes each. It is believed that this multiplicity reflects a variety of functions. In fact, not all transglycosylases are essential. As an example, in *E. coli* both the class A PBP1C and the Mgl can be deleted without noticeable harm to the cell. However either PBP1A or PBP1B must be present. It has been proposed that nonessential transglycosylases could be involved in localized reorganizations of the cell sacculus required for the assembly of envelope-spanning complexes, such as flagella, and for specialized transport processes through the envelope.

Cross-Linking of Glycan Chains

In the last step, peptidoglycan strands synthesized by glycosyl transferases must be incorporated into the macromolecular, net-like structure of the sacculus. This is the role of the peptidyl transferase activities (D,D-transpeptidase) displayed by both, class A and class B HMW-PBPs.

Transpeptidases cross-link stem peptides in the sacculus to the stem peptides in the nascent glycan strands which, consequently, become part of the sacculus proper (**Figure 4**). The canonical D,D-transpeptidation reaction consists in the transfer of the D,D-peptide bond linking the C-terminal D-Ala-D-Ala dipeptide of a disaccharide-pentapeptide unit in one strand, the donor muropeptide, onto the free NH_2 group of the dibasic amino acid at position 3 in the stem peptide of a second (acceptor) muropeptide in a nearby strand (**Figure 4**).

The transpeptidation reaction, the target for β-lactam antibiotics, has been analyzed in detail. The reaction proceeds in two steps. First the transpeptidase domain binds the stem peptide-D-Ala-D-Ala dipeptide and through a nucleophilic attack to the amide bond by the -OH of an active site Ser residue, an acyl-enzyme complex is formed. In a second step, the newly formed ester bond between the D-Ala at position 4 of the donor and the active-site Ser, is subjected to nucleophilic attack by the free NH_2-group of the dibasic amino acid in the acceptor stem peptide, resulting in formation of a new amide bond bridging both stem peptides and regeneration of the -OH in the active-site Ser. As a result of the transpeptidation reaction, the free NH_2 of the dibasic amino acid in the acceptor moiety of the resulting cross-linked peptide becomes substituted and unable to participate in further transpeptidation reactions. Conversely, the equivalent group in the donor moiety remains free and capable to act as acceptor in a subsequent round of transpeptidation to yield higher order cross-linked muropeptides. Indeed, up to tetramers have been demonstrated in *E. coli* and in a number of both Gram-negative and Gram-positive bacteria (*S. pneumoniae*), where they are particularly abundant.

As indicated before, in most Gram-positive bacteria the stem peptide is modified by acylation with a short peptide of the non-α-NH_2 group of the dibasic amino acid. In these cases, the transpeptidation reaction proceeds as above, except that the nucleophile in the second step of the reaction normally is the N-terminal NH_2 of the interpeptide bridge.

β-Lactam antibiotics react with D,D-transpeptidases, in general D,D-peptidyl transferases, because they mimic the natural donor substrate and are recognized by the donor site in the peptidyl transferase. The β-lactam bond substitutes for the D-Ala → D-Ala amide bond and acylates the active-site serine residue yielding a stable penicilloyl-enzyme complex, which disables the enzyme.

With all likelihood D,D-transpeptidation is the universal cross-linking reaction, and the reason for the wide spectrum of β-lactam antibiotics. However, cross-linking by transfer of L,D peptide bonds has also been described in several bacteria. The dibasic amino acid at position 3 (A_2pm, L-Lys) can substitute for D-Ala at position 4 as the acyl donor in the transpeptidation reaction to yield a (3 → 3) L,D cross-link, instead of the regular (4 → 3) D,D cross-link produced by D,D-transpeptidases. In this case, the transferred peptide bond is of L,D conformation. The (3 → 3) L,D cross-bridges were originally found in *Mycobacteria*. In some species as *M. smegmatis*, they account for as much as one third of total cross-bridges. In other bacteria the proportion is highly variable. In Gram-negative bacteria, (3 → 3) L,D cross-bridges (often referred to as A_2pm → A_2pm cross-bridges) range from <1% of total cross-bridges in *Pseudomonas putida*, to about 30% in *Acinetobacter acetoaceticus* and *Proteus morganii*. Abundance of L,D cross-bridges shows a substantial variability in response to environmental conditions, with a tendency to increase under stress conditions. At present, there is no clear understanding of the biochemistry of (3 → 3) L,D cross-links. It is generally accepted that synthesis occurs by a mechanism formally similar to D,D-transpeptidation, except for the nature of the donor peptide bond. Such a mechanism would be catalyzed by L,D-transpeptidases. In fact, L,D-transpeptidases have been described and partially characterized in some bacteria (*Enterococcus faecium*, *E. coli*). An important feature of L,D-transpeptidases is that because of the change in stereo specificity, they are refractory to inhibition by β-lactam antibiotics. Indeed, L,D-transpeptidases have been associated with β-lactam resistance in *E. faecium*.

Class A versus Class B HMW-PBPs

As indicated before, both classes have D,D-transpeptidase activity, but only class A are bifunctional enzymes. Therefore, while class A are able to both polymerize Lipid II into strands and incorporate the strands into the sacculus, class B are limited to the cross-linking of peptidoglycan strands necessarily fed to them by ancillary transglycosylases. However, in all bacteria where PBPs could be well studied class B PBPs are essential, and key enzymes for proper morphogenesis. For instance, PBP2 of *E. coli* is strictly required for longitudinal growth of the sacculus, and PBP3 is responsible for the synthesis of the septum when cells divide. Furthermore, it has been shown that PBP2 is by itself able to promote substantial growth of the sacculus under specific conditions. A possible interpretation of available information, by no means complete, is that class A enzymes provide bulk-synthetic capacity and class B enzymes direct the very final step of the process, fine-tuning incorporation of new material as to ensure proper shape is generated. Whether class A are feeders for class B or class B are associated to monofunctional transglycosylases (Mgt) is under discussion. However, the fact that at least in *E. coli*, Mgt is dispensable but a class A enzyme must be active for the cells to grow, favors class A and class B enzymes teaming together.

Brake to Make: Peptidoglycan Hydrolases

Enzymes capable of cleaving specific bonds in the sacculus are expected to play crucial roles to permit expansion of the whole structure. These enzymes are generally referred to as murein, or peptidoglycan, hydrolases. Some, but not all, cleave bonds that affect the physical continuity of peptidoglycan. Enzymes able to split cross-links or glycan strands can weaken the sacculus beyond a point when it is not more able to withstand the turgor pressure of the cell, and cell lysis would ensue. Therefore, murein hydrolases are dangerous enzymes for the cell, which must subject them to very stringent control. The capacity of some of them to cause cell lysis when control mechanisms are upset, by antibiotics for instance, led to the designation of ‘autolysins’. The number and variety of murein hydrolases is really staggering. Specific hydrolases exist for each and every covalent bond in peptidoglycan. Each bacterial species studied showed a wide repertory of hydrolases, and very often a high degree of redundancy for at least some activities. To give a taste, in *E. coli* not less than five different hydrolytic activities, represented by not less that 16 different proteins, have been described. Peptidoglycan hydrolases can be roughly classified according to their substrate in peptidases and glycosylases. The former cleave bonds in the stem peptide and the later on the glycan strand.

An important group of peptidases comprises the D,D- and L,D-endopeptidases and carboxypeptidases, widespread, and redundant in most bacteria. D,D-endopeptidase and D,D-carboxypeptidase activities are peptidyl transferases associated to low molecular weight PBPS. D,D-endopeptidases cut the cross-bridges attaching nearby glycan strands to each other by cleaving the amide bond between the D-Ala at position 4 of the donor stem peptide and the dibasic amino acid on the acceptor stem peptide. Thus a D,D-endopeptidase reverts the action of a D,D-transpeptidase. D,D-carboxypeptidases remove the C-terminal D-Ala from the stem peptides, which become shortened to tetrapeptides. Both sets of enzymes are generally dispensable, but likely relevant for proper morphogenesis. Impairment of their activity in *E. coli*, for instance, leads to severe morphological abnormalities as branching. The catalytic mechanism of these enzymes is similar to HMW-PBPs. In the case of D,D-transpeptidases, the donor amino acid is the D-Ala at position 4 in the donor side of the cross-bridge, while for D,D-carboxypeptidases is the equivalent D-Ala in a noncross-linked stem peptide. Upon formation of the acyl-enzyme complex, the former acceptor moiety is released and the NH_2 group of the acceptor amino acid is regenerated. The ester bond between the donor peptide and the active-site serine is then subjected to nucleophilic attack by a molecule of water releasing the free peptide and regenerating the active-site amino acid. Because of their PBP character, most DD endopeptidases and carboxypeptidases are inhibited by β-lactams. However, in some bacteria as *E. coli*, penicillin-insensitive D,D-endopeptidases (MepA) have been also identified. The MepA type endopeptidases are metallopeptidases completely unrelated to the penicillin-sensitive enzymes. L,D-endopeptidases and

L,D-carboxypeptidases have similar activities to their D,D-counterparts on peptide bonds of L,D-stereochemistry, and in most cases are believed to proceed through similar catalytic mechanisms.

A second very relevant set of murein peptidases is composed by the *N*-acetylmuramyl-L-alanine amidases. These enzymes cleave the amide bond between the lactyl group in MurNAc and the first amino acid (L-Ala) in the stem peptide. As a result of their activity, stem peptides are released from the peptidoglycan strands. Enzymes with this activity are quite heterogeneous, in function and structure and still not well characterized. Amidases are widespread, and very often highly redundant. In *E. coli* at least five molecular species present this activity. In some Gram-positive bacteria, amidases often present additional domains able to recognize and bind to specific motifs in cell wall polymers, as the teichoic and lipoteichoic acids. Recognition of these domains might be required for enzyme activation as is the case for *S. pneumoniae* LytA, which has to recognize lipoteichoic acid choline to become active.

Among the glycosylases, the family of lytic transglycosylases is predominant. These enzymes catalyze the intramolecular transfer of a MurNAc (β-1 $\rightarrow$ 4) GlcNAc glycosidic bond in a peptidoglycan strand onto the 6-OH of the MurNAc residue, cleaving the glycan strands and generating (1 $\rightarrow$ 6) anhydro MurNAc-containing muropeptides. Both endo- and exolytic transglycosylases have been identified. Exolytic enzymes are processive enzymes that degrade peptidoglycan strands from one end (GlucNAc) releasing a monomer at a time and shortening the strand by one unit. Endolytic enzymes randomly cleave any glycosidic bond in the glycan strand cutting it into a number of pieces.

N-acetylmuramidases (as lysozymes) and *N*-acetylglucosaminidases are the more frequent glycosyl hydrolases. The later are often predominant in some Gram-positive species, as *S. aureus*, where they are definitely involved in the morphogenetic pathway. However, both classes of enzymes seem to be mostly implicated in degradative processes as autolysis, peptidoglycan turnover, and even depredation (many bacteria release enzymes able to degrade peptidoglycan of niche-sharing species) than on biosynthetic pathways.

Physiology of Peptidoglycan Hydrolases

For the bacterial cell to grow the sacculus has to expand accordingly. Because the sacculus is a covalently closed structure just binding (cross-linking) new peptidoglycan strands to the sacculus is not enough to promote expansion. Indeed it would only lead to thickening of the wall. To enlarge a closed net bag, meshes have to be cleaved to permit insertion of new material between the existing netting. However, the multiplicity of murein hydrolases as well as the high redundancy of many of them, made investigation of their functionality a particularly frustrating business. The key assumption, growth of the sacculus must be strictly dependent on the activity of a particular murein hydrolase, has not been demonstrated up to now. Inactivation of single murein hydrolases very seldom results in a detectable phenotype. Even multiple mutants often retain the ability to grow in size normally. That the key hydrolase for enlargement of the cell wall has yet to be found, is possible but unlikely. Therefore, either murein hydrolases are able to functionally compensate for each other, irrespective of the differences in substrate specificity and catalytic mechanism, or enlargement of the sacculus proceeds through a yet unknown murein hydrolase-independent mechanism. Indeed, an alternative mechanism based on a peptidyl transfer reaction has been proposed. In this mechanism, a putative enzyme would transfer peptide bonds from the cross-bridges in the sacculus onto the dibasic amino acid of stem peptides in the incoming peptidoglycan strands. The reaction would formally be a transpeptidation and could, in theory, be carried out by the HMW-PBPs themselves, at least when the transferred bonds were of D,D-conformation.

Participation of murein hydrolases in the elongation of the sacculus might be in doubt, but their relevance for cell division is undeniable. As a matter of fact, the most recurrently displayed phenotype in hydrolase mutants is a defective cell division. Both in Gram-negative and Gram-positive bacteria specific hydrolases (AmiA,B,C in *E. coli*, LytA in *S. penumoniae*, Atl in *S. aureus*) are required for resolution of the septal peptidoglycan. Impairment of the corresponding enzymes results in chained cells, which are unable to split their peptidoglycan septa to finish division.

The situation, therefore, remains uncertain as to the real mechanism(s) responsible for incorporation of nascent peptidoglycan into the sacculus. Actually, available data would support coexistence of two mechanisms, one for cell enlargement and another one for septation.

Postinsertional Metabolism of Peptidoglycan

The sequence of events, schematically presented above, end up with the incorporation of new peptidoglycan into the sacculus and the corresponding expansion of the later. But this is by no means the end of peptidoglycan metabolism. As a matter of fact, the sacculus is subjected to a constant and complex reorganization. At least three processes have been documented in the *E. coli* model system: maturation, turnover, and growth-state adaptation.

Peptidoglycan Maturation

Investigation of the evolution of new peptidoglycan as it ages indicates that new and old murein differ to a considerable extent at least in *E. coli*, *B. subtilis*, and *Lactococcus lactis*. In the *E. coli* case, maturation is characterized by elimination of the D-Ala at position 5 of the stem peptides, by a notable increase in cross-linking, and a reduction in the mean length of peptidoglycan strands. The enzymology of maturation is still poorly understood. Low-molecular-weight PBPs are definitely involved, but the molecular identity of many of the activities suspected to intervene in the process remains unknown.

Peptidoglycan Turnover and Recycling

The phenomenon of murein turnover, that is shedding of peptidoglycan muropeptides from the sacculus in the course of normal growth, was first described in Gram-positive organisms (*Bacillus megaterium*, *B. subtilis*, *L. monocytogenes*, *S. aureus*, *Lactobacillus acidophilus*, etc.). Because most Gram-positive bacteria lack a permeability barrier external to the sacculus, turnover products are normally released into the growth medium, making detection relatively straightforward. Demonstration in Gram-negative bacteria (*E. coli*, *Salmonella enterica*, *N. gonorrhoeae*, *Neisseria subflava*) took more effort because turnover is often coupled to very efficient recycling of the released muropeptides, which are transported back to the cytoplasm and then enter the biosynthetic pathway. The magnitude of the turnover process is variable from species to species and may depend on growth conditions. In most instances it is quite relevant. Between 20 and 50% of total peptidoglycan is turned over each generation in those species where it has been measured as *E. coli* (30–40%) *B. subtilis* (50%), *B. megaterium* (50%), *S. aureus* (25%), *N. gonorrhoeae* (20–50%). These high turnover rates mean that peptidoglycan has to be synthesized and incorporated into the sacculus at a rate considerably higher than growth rate. Turnover is often considered a requirement for cell expansion, and to be associated to damage assessment and defense mechanisms.

Turnover is an enzymatically catalyzed process mediated by peptidoglycan hydrolases. The main turnover products in many Gram-negative bacteria are $(1 \rightarrow 6)$anhydro-MurNAc containing monomeric muropeptides, the product of lytic transglycosylases. In Gram-positive bacteria turnover products are more diverse; in some, like *B. subtilis,* turnover is mediated by a single hydrolase, a MurNAc-L-alanine amidase, but often shed out products are the result of a more complete degradation of muropeptides involving several enzymes.

Interestingly, peptidoglycan turnover is an important virulence factor in some pathogens, as *N. gonorrhoeae* and *Bordetella pertussis*. Both species have intensive turnover and release considerable amounts of GlucNAc-$(1 \rightarrow 6)$ anhydro MurNAc-L-Ala-D-Glu-A$_2$pm-D-Ala, into the extracellular environment. This muropeptide has a strong cytotoxic action on ciliated epithelia and is identical to the tracheal cytotoxin (TCT) of *B. pertussis*, and the peptidoglycan-derived cytotoxin (PGCT) of *N. gonorrhoeae*. Furthermore, peptidoglycan fragments have strong immunogenic and mitogenic activities influencing the course of infection.

Turnover products are often recycled, in particular in Gram-negative bacteria where released muropeptides accumulate in the periplasmic space. Several recycling pathways apparently exist. In *E. coli* the major route for recycling is via the transporter protein AmpG, a transmembrane protein acting as a specific permease for intact muropeptides. AmpG transports GlucNAc-$(1 \rightarrow 6)$anhydro MurNAc-L-Ala-D-Glu-A$_2$pm, the main turnover product in this bacteria, to the cytoplasm. There, it is further degraded by a β-*N*-acetyl-glucosaminidase, which splits the disaccharide releasing GlucNAc and $(1 \rightarrow 6)$anhydro MurNAc-L-Ala-D-Glu-A$_2$pm and a MurNAc-L-alanine amidase (AmpD), which breaks up the later fragment into $(1 \rightarrow 6)$ anhydro MurNAc and the stem tripeptide. AmpD has a strict requirement for $(1 \rightarrow 6)$ anhydro MurNAc, and therefore is inactive against the cytoplasmic precursors present in the cytoplasm as UDP-MurNAc-pentapeptide. Interestingly, the tripeptide released by AmpD is not further degraded. Instead, it is directly hooked onto a molecule of UDP-MurNAc by the enzyme Mlp (UDP-*N*-acetylmuramate:L-alanyl-D-glutamate-*meso*-diaminopimelate ligase) in the presence of ATP. Although both Mlp and MurC add amino acids to UDP-MurNAC, they could neither compensate for each other, nor accept each other peptide substrates. Mlp paralogues have been identified in other bacteria (*Haemophilus influenzae*), indicating that similar pathways could be widespread.

Peptidoglycan recycling has been recently associated with induction of chromosomal β-lactamases of the AmpC type in Enterobacteria. β-Lactamases are the major bacterial defense mechanism against β-lactams. Enterobacterial AmpC are inducible enzymes, and induction requires an operational peptidoglycan recycling pathway. Presence of antibiotics cause increased intracellular levels of $(1 \rightarrow 6)$ anhydro MurNAc-L-Ala-D-Glu-A$_2$pm, which binds to AmpR, a regulatory protein of the *amp* operon, and induces synthesis of β-lactamase. Therefore, turnover and recycling might also play a sensory function to detect cell wall-damaging agents.

Adaptive Modifications of Peptidoglycan

The composition and structure of peptidoglycan changes in response to environmental variations, in particular during the transitions in growth phase. Information is still limited to a few systems (*E. coli*, *S. enterica*, *H. pylori*, and *B. subtilis*) but it may well be a widespread phenomenon. Transition into stationary phase is a complex adaptive process, which involves a global reorganization of cell metabolism and very often specific morphological changes. In *E. coli* transition into stationary phase triggers a full set of modifications both in the characteristics of peptidoglycan itself and in the complement of enzymes involved in peptidoglycan metabolism, PBPs, and peptidoglycan hydrolases. Compared with exponentially growing cells, peptidoglycan from stationary-phase *E. coli* is about 50% more cross-linked, has close to double amount of bound lipoprotein. and is made up of, on average, 30% shorter glycan chains. These modifications affect total peptidoglycan and are thought to require activity of specific enzymes. Interestingly, *E. coli* cells in the so-called ‘viable but nonculturable’ state, a condition though to be clinically relevant in pathogenic bacteria, display similar alterations in their peptidoglycan. In *B. subtilis* peptidoglycan, cross-linking is also higher in stationary-phase cells. Increased cross-linking seems to be a rather general feature of nongrowing cells, and has been detected in all cases studied.

Stationary-phase cells often display specific modifications in both biosynthetic and hydrolytic peptidoglycan enzymes. Activation of specific hydrolases is in fact thought to be critical for induction of the autolytic response of many bacteria, as *S. pneumoniae*, to prolonged starvation and other adverse conditions as the presence of antibiotics.

Stationary-phase *E. coli* cells retain a considerable peptidoglycan biosynthetic activity. This activity is apparently associated to turnover and could account for renovation of about 20% of the cell peptidoglycan per hour. Interestingly, the class B PBP2 seems to be the key synthase in this process. The relevance of stationary-phase synthesis has not been asserted yet; however, an involvement in cell survival seems most likely.

Biophysical Properties of Peptidoglycan Sacculi

Thickness of Sacculi

As indicated earlier, bacteria fall in two broad categories, Gram-positive and Gram-negative, according to the organization of their cell envelopes. The former group displays a relatively thick peptidoglycan sacculus with covalently linked accessory polymers, while the later normally exhibits very thin sacculi devoid of accessory polymers. Precise measurement of the real thickness of sacculi has proven to be a rather complex task. Classical electron microscopy techniques had profound effects on the apparent thickness of sacculi in thin sections. Introduction of cryo-electron microscopy of frozen hydrated samples and atomic force microscopy recently led to a precise estimation of peptidoglycan thickness. In Gram-negative bacteria, results were unexpected because sacculi from *E. coli* and *Pseudomonas aeruginosa*, two species with identical peptidoglycan, gave different values. Cryo-electron microscopy of frozen hydrated cells gave peptidoglycan thickness values of 6.35 ± 0.53 nm for *E. coli* and 2.41 ± 0.54 for *P. aeruginosa.* Atomic force microscopy produced very close values, 6.0 ± 0.5 and 3 ± 0.5 nm for hydrated *E. coli* and *P. aeruginosa* sacculi, respectively. Concordance of both techniques is reassuring, but clearly indicates that the three-dimensional organization of peptidoglycan in sacculi of Gram-negative bacteria is more variable than previously expected. Small-angle neutron scattering measurements indicate that in *E. coli* the sacculus in not homogeneous in thickness; about 70% of total peptidoglycan is arranged in a monolayer, but up to 30% is in triple-layered domains. Unfortunately, the technique does not resolve the localization of the multilayered regions. Species to species variations in the abundance and distribution of multilayered regions could well explain the different apparent thickness in thin sections. In any case, these results seriously question the classical view of Gram-negative cell walls as a strict monolayer.

Sacculi from Gram-positive bacteria are undoubtedly multilayered, and variable in thickness. Application of advanced techniques, however, revealed a bipartite organization of the cell wall. In all species studied there is an inner zone of low density, the inner wall zone (IWZ), about 16 nm thick, apparently impoverish in polymeric materials, surrounded by the outer wall zone (OWZ) representing the polymeric peptidoglycan-teichoic acid complex. The OWZ is often in the 15–30 nm range, but is quite variable.

Elasticity of Sacculi

Purified peptidoglycan sacculi behave like an elastic fabric. Available measurements indicate that surface of *E. coli* sacculi can reversibly expand and shrink by a factor of 3 without ruptures. Different approaches support the idea that sacculi are under dynamic stress in the living cell, expanding, and shrinking in response to variations in turgor pressure. In the growing *E. coli* cell, sacculi appear to be stretched to about 140% of their surface in the relaxed (no turgor pressure) state.

A most interesting observation is the anisotropic character of elasticity in sacculi. Sacculi from rod cells are significantly more deformable along the longitudinal axis of the cell than in the transversal axis. Measurements in the atomic force microscope gave elastic moduli of 2.5×10^7 and $4.5 \times 10^7\,\mathrm{N\,m^{-2}}$ in the respective directions (smaller elastic modulus means greater elasticity). These measurements are in agreement with the observation that changes in cell volume due to osmotic challenge are mainly due to elongation of the cells, while diameter remains nearly constant. In macromolecular peptidoglycan, peptide bridges can be stretched much further than the virtually inextensible glycan backbones. For this reason, elastic anisotropy is considered as a strong indication for a preferential orientation of the glycan strands perpendicular to the long axis of the cell. However, X-ray diffraction and infrared spectroscopy indicate that the sacculus is far from a regularly ordered structure. Therefore, glycan chains may well be organized with a preferential orientation, but the layout of the sacculus is far from a highly ordered structure.

Permeability of Sacculi

Bacteria secrete numerous macromolecules, which must necessarily cross the sacculus on their way to the external environment. Moreover, assembly of supramolecular complexes spanning the cell envelope as flagella or type III protein secretion systems, also have to deal with the presence of the sacculus. Therefore, it is relevant to know whether and how the structure of the sacculus, or the activity of particular enzymes, permits traffic and assembly of macromolecules.

Experimental data on the porosity of sacculi are scant and limited to *E. coli* and *B. subtilis*. Interestingly, pores in purified (relaxed) sacculi from both organisms are of similar average size, with estimated radii of 2.06 nm for *E. coli* and 2.12 nm for *B. subtilis*. This size limits free passage of globular proteins to molecular weights about 22–25 kDa in the relaxed state of peptidoglycan, which could go up to around 50 kDa for fully stretched sacculi. Larger molecules could pass through the sacculus either in nonfolded or partially folded forms, or through specialized mechanisms involving local peptidoglycan rearrangements. Assembly of supramolecular structures almost certainly requires such rearrangements. For instance, the flagellar rod has an average diameter of about 11 nm, much wider than peptidoglycan expected discontinuities. Participation of murein hydrolases in these rearrangements has been substantiated in a number of bacteria. In *C. crescentus* the transglycosylase PleA is required for assembly of both pili and flagella at the cell pole. In *S. enterica* and *Rhodobacter sphaeroides* flagellar proteins with muramidase activity (FlgJ) are required for flagellar assembly, which otherwise stalls at the M ring stage. Paralogues to hydrolytic enzyme genes have been found in operons coding for flagella, pili, and type III secretion systems, indicating that participation of peptidoglycan hydrolases in assembly processes might be a general mechanism.

Topological Heterogeneity in Sacculi

The existence of peptidoglycan patches with different properties in sacculi was first detected in *B. megaterium*. The polar caps of the rod-like cells were shown to turnover far more slowly than the lateral cell wall. More recently, it has been shown that the peptidoglycan at the polar caps of *E. coli* sacculi is metabolically inert (IPG). That is, neither new precursors are inserted into these regions nor are they turned over. As a consequence IPG has a very long life. As polar regions are the product of a previous septation event, IPG can be considered the final product of such a septation event. The mechanism underlying generation of IPG is unknown. Nevertheless, at least three proteins, PBP5, BolA, and FtsZ, have been shown to affect proper distribution of IPG in *E. coli*. All three proteins are involved in the morphogenetic pathway. Therefore, it seems that proper differentiation of IPG regions might be an important process for the cell. At present, no specific structural or chemical features have been found in IPG.

Concluding Remarks

The role of peptidoglycan as a reinforcement for the bacterial cell envelope was firmly established more than 60 years ago and the basics of its structure and metabolism were worked out soon after. However, the full complexity of the bacterial sacculus become apparent only recently, hand in hand with the impressive advances in the field of bacterial division differentiation and morphogenesis. The structure once thought to be a rigid and fundamentally inert structure is showing a totally unexpected degree of complexity and dynamism at all imaginable levels. Further research on the structural properties, biochemistry, and physiology of this fascinating macromolecule will undoubtedly help us to better understand bacterial life.

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Photosynthesis: Microbial

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Glossary

anoxygenic photosynthesis A type of photosynthesis performed by bacteria that do not use water as the electron donor for carbon fixation, and hence do not liberate oxygen.

antenna A network of closely spaced pigment molecules that captures light energy and transfers it efficiently to the reaction center.

chlorosome An extensive antenna system consisting of bacteriochlorophyll *c*, *d*, and *e* found in green sulfur bacteria.

iron–sulfur cluster A redox cofactor consisting of covalently linked iron and sulfur atoms that is involved in one-electron transfers.

reaction center A membrane-embedded pigment–protein complex responsible for transferring electrons across the photosynthetic membrane.

thylakoid A membranous structure wherein all the light-dependent processes of photosynthesis occur.

water-splitting complex A cluster of four manganese atoms and one calcium atom that oxidatively splits water, producing electrons for carbon fixation.

Abbreviations

3PG	3-phosphoglycerate
CBB	Calvin–Benson–Bassham
Chl	Chlorophyll
DHAP	dihydroxyacetone phosphate
EXAFS	extended x-ray absorption fine structure
F6P	fructose-6-phosphate
FBP	fructose-1,6-biphosphate
FNR	ferredoxin-NADP$^+$ oxidoreductase
G1P	glucose-1-phosphate
G3P	glyceraldehyde-3-phosphate
NADPH	nicotinamide adenine dinucleotide phosphate
PS	Photosystem
RuBP	ribulose-1,5-biphosphate

Defining Statement

The aim of this article is to move beyond the textbook equation of photosynthesis and describe the design principles behind photosynthetic electron transfer. The events that constitute a photosynthetic cycle are described in the exact order they occur, using the cyanobacterial system as a model. Photosynthesis in lesser-known phototrophs is also discussed.

Introduction

Photosynthesis is the biochemical process carried out by certain bacteria, algae, and higher plants in which light is converted into chemical bond energy. The process is crucial, since nearly all life on earth depends on sunlight either directly or indirectly for energy, food, and O_2. The advent of photosynthetic prokaryotes with the ability to consume CO_2 and produce O_2 from H_2O resulted in a hospitable environment on earth for advanced forms of life. Fossil records indicate that the first oxygenic photosynthetic bacteria appeared around 3.5×10^9 years ago. Earlier, organisms survived by anaerobic metabolism, a process that generates only a fraction of the energy produced by aerobic metabolism. It is likely that in the absence of oxygenic photosynthesis, advanced forms of life would not have emerged and only microorganisms would now exist. Today, as the primary means of carbon fixation, oxygenic photosynthesis forms one half of the energy-carbon cycle. Phototrophic organisms reduce CO_2 to carbohydrates, which are oxidized back to CO_2 by heterotrophic (as well as phototrophic) organisms. The energy released during the oxidation reaction is stored in the form of NADH and ATP, which are subsequently used for growth, metabolism, and reproduction. In addition, prehistoric plants and algae were largely responsible for the generation of the vast reserves of fossil fuels that are now being mined for their energy value. They provided a large portion of the initial biomass, which was converted into oil and coal over millions of years through pressure, heat, and microbial action.

The general process of photosynthesis is described by Van Niel's equation:

$$2H_2A + CO_2 \rightarrow 2A + CH_2O + H_2O \qquad [1]$$

where H_2A is the reductant and A is the oxidized product.

Van Niel's equation can be applied to oxygenic photosynthesis as:

$$6CO_2 + 6H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 \qquad [2]$$

Although complete, this equation belies the overwhelming complexity of the process. For example, the generation of the light-induced charge-separated state and its subsequent stabilization over time requires a large number of pigments and cofactors arranged in a specific protein environment. The splitting of H_2O into O_2 is extremely difficult to replicate in the laboratory, yet plants and cyanobacteria perform the task repeatedly with seeming ease. The conversion of CO_2 into sugars is another intricate process that requires an extensive set of physical and chemical reactions to occur in a highly coordinated fashion.

In this article, we will expand on this simple equation. In addition to describing the general design principles behind the sophisticated biomachinery involved in photosynthesis, we will provide structural and functional details, placing special emphasis on light-induced electron transfer in aerobic and anaerobic organisms.

Historical Perspective

The first experiments on photosynthetic organisms were performed in the 1770s when Joseph Priestley showed that plants were capable of generating a gas that could support combustion. Building on his work, Jan Ingenhousz established that sunlight was required, and Jean Senebier and Nicolas Theodore de Saussure demonstrated the indispensability of CO_2 and H_2O. In 1845, Julius Robert von Meyer postulated that plants convert light into chemical energy during photosynthesis. Early scientists believed that the O_2 was produced from the splitting of CO_2, and it was not until the 1930s that Cornelius van Niel proposed, correctly, that H_2O was the source of O_2. It is interesting that 75 years later the exact biochemical mechanism of H_2O splitting remains to be elucidated.

Photosynthesis research has had its share of Nobel laureates. Melvin Calvin won the chemistry prize in 1961 for identifying most of the intermediates in the conversion of CO_2 into carbohydrates. Peter Mitchell was the sole recipient of the chemistry award in 1978 for his work on the chemi-osmotic theory of proton translocation. Johann Deisenhofer, Robert Huber, and Hartmut Michel won the chemistry prize in 1988 for solving the first crystal structure of a photosynthetic reaction center. Rudolph Marcus's investigation of the factors guiding electron transfer in chemical systems remains the paradigm for theoretical calculations of electron transfer in photosynthetic reaction centers. He was awarded the Nobel Prize in chemistry in 1992. More recently, Paul Boyer and John Walker were awarded the prize in chemistry in 1997 for elucidating the enzymatic mechanism underlying the synthesis of ATP.

Artificial photosynthesis has seen a recent spurt of activity, largely due to an increased awareness of the depletion of fossil fuel reserves and the effect of their combustion products on the earth's climate. The goal is to synthesize inexpensive and long-lasting organic and inorganic molecules that convert light into chemical energy, thereby mimicking the basic process of photosynthesis. This has brought new disciplines such as material science and bioengineering into photosynthesis, making the field truly interdisciplinary.

Classification of Photosynthetic Organisms

There exist five bacterial phyla with members capable of chlorophyll-based phototrophy: Firmicutes, Chloroflexi, Chlorobi, Proteobacteria, and Cyanobacteria. With the recent discovery of *Chloracidobacterium thermophilum*, Acidobacteria have become the sixth known phylum to carry out the process of photosynthesis.

All photosynthetic organisms can be classified as either oxygenic or anoxygenic. Oxygenic phototrophs employ H_2O as the source of electrons and liberate O_2 as the by-product. Anoxygenic phototrophs derive their electrons from organic or inorganic molecules, and hence they do not evolve O_2. Of the five well-established phototrophic bacterial phyla, only the Cyanobacteria are capable of performing oxygenic photosynthesis. In addition, all eukaryotic phototrophs such as higher plants and algae, which evolved later than cyanobacteria, produce O_2 during photosynthesis.

The remaining four phyla include anaerobes such as the purple nonsulfur bacteria, purple sulfur bacteria, green sulfur bacteria, and heliobacteria, which survive only under low concentrations of O_2. The recently discovered Acidobacteria have been reported to live under oxic conditions, although a detailed physiological characterization of this organism remains to be carried out.

We will discuss oxygenic photosynthesis first, using cyanobacteria as the model organism. Cyanobacteria are photosynthetic prokaryotes that are found in every conceivable habitat from oceans to fresh water to soil. These Gram-negative bacteria are responsible for generating the majority of the O_2 in the earth's atmosphere. The most widely used cyanobacterial strains for current experimental research are *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, and *Thermosynechococcus elongatus*.

In cyanobacteria, photosynthesis is associated with a well-organized system of internal membranes in the cytoplasm. These are called thylakoids, from the Greek word *thylakos* meaning sac. These membranes are highly folded, allowing the cell to pack a large amount of surface area into a small space. The interior space enclosed by the thylakoid membrane is termed the lumen and the matrix surrounding the thylakoids is termed the stroma. The thylakoids are home to the integral membrane protein complexes that are involved in the light reactions of photosynthesis.

Eukaryotic organisms such as higher plants and algae conduct photosynthesis in membrane-bound organelles called chloroplasts. They consist of an outer, freely permeable membrane and a selectively permeable inner membrane that encloses the stroma. The sac-like thylakoids immersed in the stroma are similar in organization to the comparable membranes in cyanobacteria. Chloroplast thylakoids, however, tend to form well-defined stacks called grana, which are connected to other stacks by intergrana thylakoids called lamellae. It is widely thought that chloroplasts evolved from an endosymbiotic relationship of a heterotrophic prokaryote with a cyanobacterium.

The Constituent Processes of Photosynthesis

Eqn [1] is the end product of a large number of events that occur during a typical photosynthetic cycle. The basic processes that constitute oxygenic photosynthesis are:

- Absorption of light by pigment molecules and transfer of the excitation energy to two reaction centers, Photosystem II (PS II) and Photosystem I (PS I).
- Light-induced transfer of an electron across the photosynthetic membrane and splitting of H_2O into O_2 by PS II.
- Light-induced excitation and transfer of an electron across the photosynthetic membrane, generating reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) by PS I.
- Production of ATP using the proton gradient generated across the membrane from both H_2O splitting and electron transfer through the cytochrome b_6f complex.
- Conversion of CO_2 into carbohydrates using ATP and the reducing power of NADPH.

The division of photosynthetic labor is relatively straightforward. All the light reactions occur within or on the thylakoid membrane. The ATP and NADPH produced by the light reactions are released into the stroma where the dark reactions of CO_2 fixation are carried out. We focus first on the overall design philosophy of the process of converting light to stable chemical energy.

Absorption and Transfer of Light Energy

The Light-Absorbing Chromophores

Photosynthesis in cyanobacteria and plants is driven by light in the visible (380–750 nm) region of the electromagnetic spectrum. Phototrophic organisms such as purple bacteria, green sulfur bacteria, and heliobacteria extend this region to the near-infrared so as to exploit unique ecological niches. All of this makes evolutionary sense as the majority of the sun's energy that reaches the earth's surface lies in this range. Ultraviolet radiation and far-infrared radiation are both limited in amount; also, the former is too energetic and is capable of breaking chemical bonds, while the latter contains insufficient energy to be useful for most photochemical processes.

Primary chromophores

Photosynthetic organisms use a range of chromophores to efficiently capture photons in the visible and near-IR regions. The most abundant chromophore involved in photosynthesis is chlorophyll, a molecule structurally similar to, and produced by, the same metabolic pathway as porphyrin pigments such as heme. The basic structure of the chlorophyll molecule is a chlorin ring coordinated to a central magnesium atom (**Figure 1**). The addition of a long phytol tail makes chlorophyll insoluble in water. There are four common types of chlorophyll molecules in photosynthetic organisms, named chlorophyll *a*, *b*, *c*, and *d*. Their overall structure is similar, with minor changes in the side-chain groups that result in slightly different absorption spectra (**Figure 2**). Cyanobacteria employ chlorophyll *a*, while plants utilize both chlorophyll *a* and *b*. Some species of algae contain chlorophyll *c*, and a few species of cyanobacteria contain chlorophyll *d*. Chlorophylls absorb primarily in the blue and red regions of the visible spectrum and have a high molar extinction coefficient. They have an inherently high fluorescence yield, which guarantees a long-lived excited singlet state, making them the ideal chromophore.

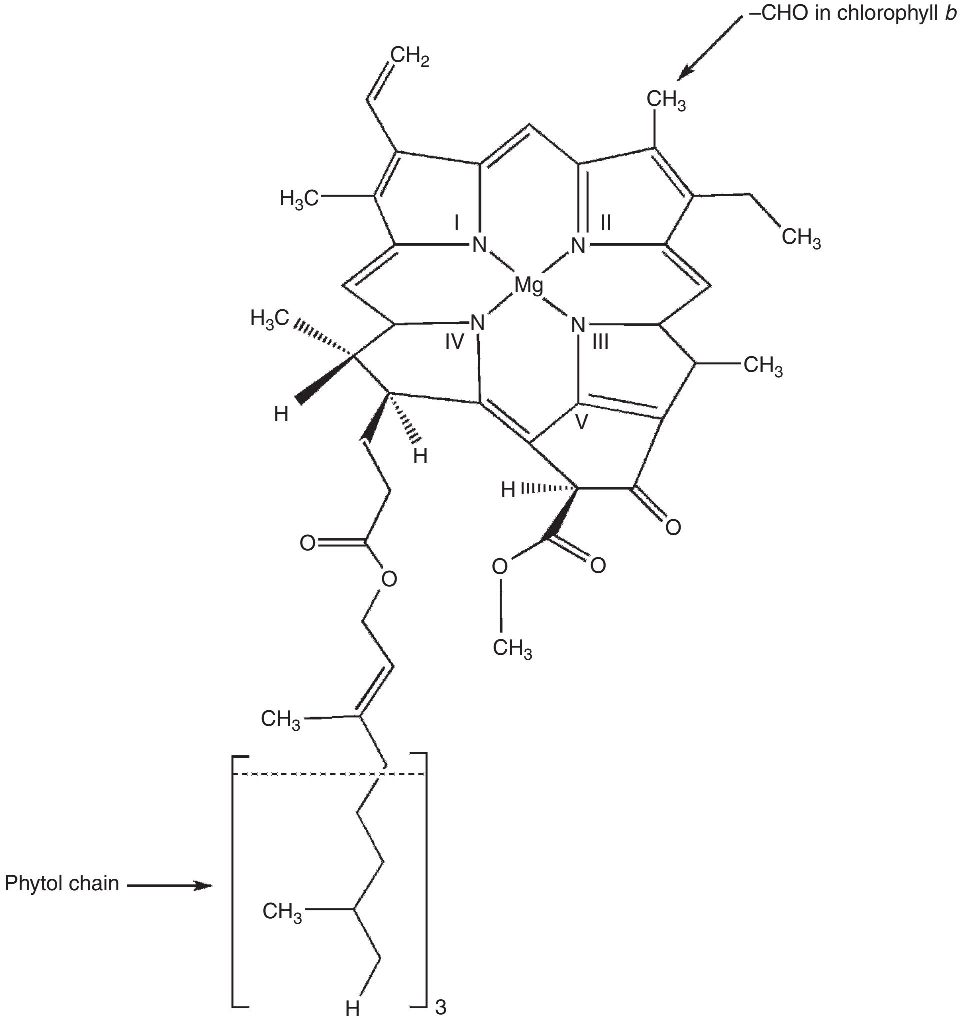

–CHO in chlorophyll *b*
CH2
CH3
H3C
I
II
N
N
Mg
N
N
IV
III
CH3
H3C
H
H
CH3
V
H
O
O
O
O
O
CH3
CH3
Phytol chain
CH3
H
3

Figure 1 Chemical structure of chlorophyll *a*. Chlorophyll *b* has a –CHO group instead of the –CH_3 group in ring II.

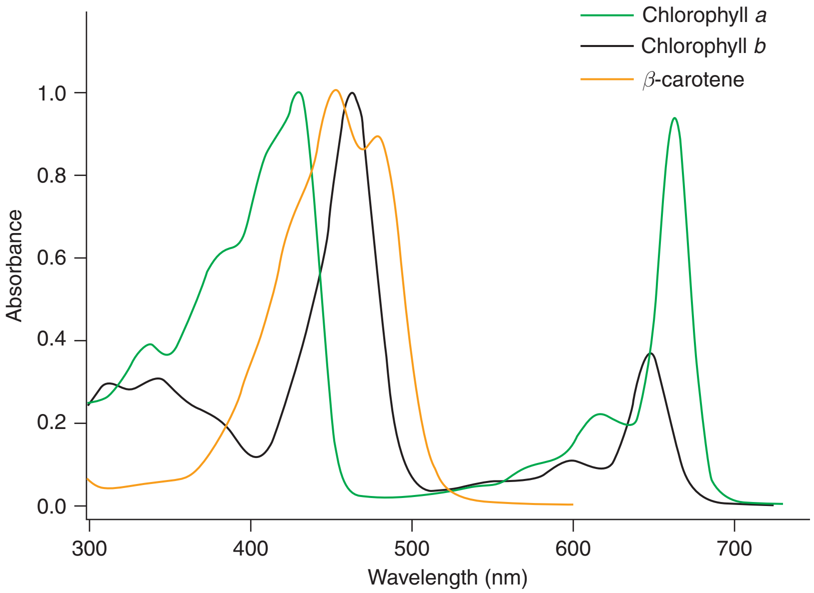


Figure 2 The absorption spectra of chlorophyll *a*, chlorophyll *b*, and *β*-carotene in solution.

Accessory chromophores

Besides containing chlorophylls, photosynthetic organisms contain accessory pigments that extend the range of absorbed wavelengths. Carotenoids are the main accessory pigment found in cyanobacteria, algae, and higher plants. They belong to the tetraterpenoid family, that is, contain 40 carbon atoms, and absorb light in the 400–500 nm region. Structurally, these compounds are composed of two small six-carbon rings connected by a polyene chain of carbon atoms. They are insoluble in water and are normally attached to proteins that are attached to the membrane. There are over 600 types of carotenoids, which are classified as either carotenes or xanthophylls. Carotenes consist exclusively of carbon and hydrogen, while xanthophylls also contain oxygen. The most abundant carotenoid in cyanobacteria is β-carotene, which is the same pigment that gives carrots its distinctive color (**Figure 3**). In addition to functioning as an accessory pigment, carotenoids play a vital role in dissipating excess light energy, which would otherwise lead to the generation of superoxide radicals. These radicals are highly reactive to chemical bonds and could be potentially lethal to the cell if left unchecked.

Cyanobacteria and certain types of algae contain additional pigments called phycobilins, which absorb light between 500 and 650 nm. Phycobilins consist of an open chain of four pyrrole rings and are water-soluble. They are attached to proteins termed phycobiliproteins and they pass on the absorbed light energy to nearby antenna chlorophyll molecules.

Plants and cyanobacteria therefore use a combination of chlorophylls and accessory pigments to effectively blanket a large majority of the visible spectrum. Both appear dark green or blue-green because the few photons that are not absorbed lie between the blue and red regions of the spectrum.

The Light-Gathering Structures and Resonance Energy Transfer

The task of the photosynthetic reaction center is to convert the energy stored in the excited singlet state of chlorophyll to a form useful for work. In photosynthesis, work refers to the creation of a charge-separated state consisting of a donor, D^+, and an acceptor, A^-, pair. At one extreme of time, the creation of the singlet excited state occurs within 10^{-15} s of absorbing a photon. At the other extreme, the captured light energy must be utilized within 10^{-8} s, otherwise the energy will be lost as heat or fluorescence as the excited state decays. The generation of the charge-separated state must occur within this window of time.

A network of closely spaced chlorophyll molecules, termed the antenna system, absorbs the photon and the resulting excited state migrates to a neighboring antenna chlorophyll by a process known as resonance energy transfer. This occurs on a timescale of 10^{-12} s and is a nonradiative process. The excited state, known as an exciton, randomly wanders about the antenna system until it chances upon the specialized reaction center chlorophylls associated with PS I and PS II. The energy levels of these specialized chlorophylls are slightly lower than the antenna chlorophylls because they are in a different protein environment. This allows these specialized chlorophylls to trap the exciton and use it to create a charge-separated state. In most photosynthetic reaction centers, this state is generated within 10^{-10} s following photon absorption. Accessory pigments also transmit the absorbed energy to antenna chlorophylls by a similar process of resonance energy transfer.

The Water-Splitting Complex

We now turn our attention to the source of electrons in oxygenic photosynthesis. The catalytic redox center that carries out H_2O splitting is termed the O_2-evolving complex, and is an integral component of PS II. The water-splitting reaction can be summarized by the following equation:

$$2H_2O \rightarrow O_2 + 4e^- + 4H^+ \qquad [3]$$

The 3.0 Å X-ray crystal structure of PS II from *T. elongatus* (PDB ID 2AXT), as well as extended X-ray absorption fine structure (EXAFS) studies on PS II crystals, has led to a structural model of the O_2-evolving complex (**Figure 4**). This structure is the starting point for discussion on the mechanism of O_2 evolution.

A cluster of four manganese atoms and a calcium atom is responsible for stripping four electrons from two H_2O

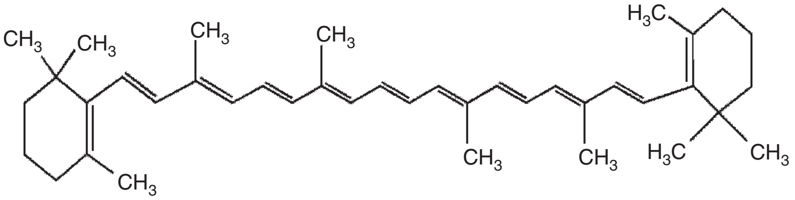


Figure 3 Chemical structure of β-carotene.

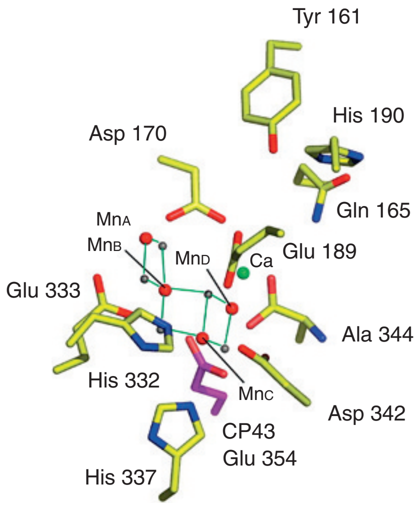


Figure 4 Proposed structure of the water-splitting complex of PS II, based on the 3.0 Å resolution X-ray crystal structure and on extended X-ray absorption fine structure (EXAFS) data. The spheres represent manganese (red), calcium (green), and the bridging oxygen ligand atoms (gray).

molecules, liberating O_2 in the process. The protons are released into the thylakoid lumen, thereby generating a portion of the pH gradient that is used to synthesize ATP.

The structural model of PS II shows the metal atoms arranged in an extended cubane structure, with three manganese and one calcium at the corners, and the fourth manganese located immediately to one side. The metal atoms are connected to each other by mono-μ-oxo, di-μ-oxo, and/or hydroxo bridges, but the amino acids that contribute the ligands are not known with complete certainty.

Since 1970, the paradigm for understanding O_2 evolution has been the S-state cycle proposed by Bessel Kok. This model includes five oxidation states (S-states) for the 4Mn–Ca cluster. The cluster is oxidized in one-electron steps from S_0 (most reduced) to S_4 (most oxidized) by a successively photooxidized reaction center chlorophyll in PS II. The 4Mn–Ca cluster thus accumulates four equivalents of oxidizing power and uses it to split two H_2O molecules. An O_2 molecule is released after the S_4 state, returning the 4Mn–Ca cluster to the S_0 state.

A large number of questions remain unsolved concerning the catalytic mechanism of water splitting. Most importantly, the S_4 state of the cycle is fleeting and has not been observed spectroscopically. This state is critical because it may be the starting point for O–O bond formation. Without knowledge of its chemistry, the precise catalytic mechanism of the water-splitting complex is difficult to formulate. The binding site of the two H_2O molecules represents another uncertainty, although a recent proposal suggests that the calcium coordinates one of the H_2O molecules, with a manganese binding the other.

The electrons from the water-splitting complex are donated to an oxidized tyrosine residue termed Tyr_Z. According to the X-ray crystal structure, the calcium atom is positioned between the 4Mn–Ca cluster and Tyr_Z. A chloride ion is bound near the water-splitting complex in the vicinity of Tyr_Z. It is believed that its role in O_2 evolution is to neutralize accumulated charge.

Transmembrane Electron Transfer

Stabilization of the Charge-Separated State

The electrons obtained from the splitting of H_2O are ultimately used to reduce CO_2. To achieve this goal, the electrons must be transferred against a highly unfavorable thermodynamic gradient from the lumenal side of the membrane to the stromal side, where the dark process of carbon fixation occurs. The electrons traverse a distance equal to the thickness of the membrane, which is around 40 Å. This distance appears short, but it is significant on the scale of an electron.

Consider a hypothetical donor–acceptor pair, D–A, where D becomes excited to D^* and donates an electron to A, thereby creating a charge-separated state D^+–A^-:

$$\text{D–A} \rightarrow \text{D}^*\text{–A} \rightarrow \text{D}^+\text{–A}^-$$

Due to their close proximity, the D^+–A^- charge-separated state is unstable and short-lived. Adding a second closely spaced acceptor molecule, A_1, can extend the lifetime of the charge-separated state:

$$\text{D–A–A}_1 \rightarrow \text{D}^*\text{– A–A}_1 \rightarrow \text{D}^+\text{–A}^-\text{–A}_1 \rightarrow \text{D}^+\text{–A–A}_1^-$$

The driving force for electron transfer is a drop in Gibbs free energy between A^- and A_1, thus altering the equilibrium constant in favor of A_1. This results in the expenditure of some of the original energy of the photon, but it is a necessary trade-off to extend the lifetime of the charge-separated state.

Adding a second closely spaced donor molecule, D_1, has a similar effect:

$$\text{D}_1\text{–D–A} \rightarrow \text{D}_1\text{–D}^*\text{–A} \rightarrow \text{D}_1\text{–D}^+\text{–A}^- \rightarrow \text{D}_1^+ - \text{D–A}^-$$

The positive charge will migrate from D^+ to D_1 due to a drop in Gibbs free energy between D_1 and D, thus altering the equilibrium constant in favor of D.

Photosynthetic complexes adopt both strategies and trade off some of the energy of the original photon to extend the lifetime of the otherwise short-lived charge-separated state.

Factors Affecting the Rate of Electron Transfer – Marcus Theory

To further understand this process, we must delve further into the factors that govern the rate of electron transfer in proteins. Marcus theory states that the rate of electron transfer between a donor and an acceptor pair depends on two factors. The first is the Frank Condon factor, which includes the change in the Gibbs free energy, the reorganization energy, and the temperature. Mathematically, it is expressed as:

$$k_{\mathrm{et}} \propto \exp\frac{(-\Delta G_0 + \lambda)^2}{4\lambda k_{\mathrm{B}} T} \qquad [4]$$

where k_{et} is the rate of electron transfer, ΔG_0 is the difference in free energy between the product and the reactant, λ is the reorganization energy, k_{B} is the Boltzmann's constant, and T is the absolute temperature. This is the equation of a parabola; as ΔG increases, the rate first increases, then attains a maximum, and finally decreases. The difference in Gibbs free energy between the product (D^+–A^-) and the reactant (D–A) translates into the thermodynamic driving force for the reaction. The reorganization energy corresponds to the amount of energy required to alter the microenvironment of the reactants before electron transfer so that it resembles the equilibrium microenvironment of the products after electron transfer. This term reflects small changes in bond lengths and reorientation of dipoles around the redox centers to reflect the new pattern of electric fields before and after the electron transfer event. It is difficult to measure experimentally and is usually assumed to be a constant 0.7 eV in proteins.

The second factor that influences the rate of electron transfer is the matrix coupling element. It relates electron transfer rate to the distance between the donor and the acceptor pairs. The following equation describes the relationship:

$$|H_{\mathrm{AB}}|^2 \propto \exp(-\beta R) \qquad [5]$$

where $|H_{\mathrm{AB}}|$ is the coupling probability between the donor and acceptor wave functions, β is a constant whose value is 1.4 Å^{-1} in proteins, and R is the edge-to-edge distance between the donor and the acceptor molecules.

In proteins, this logarithmic relationship translates to a tenfold change in the rate of electron transfer for every 1.7 Å change in distance. Accordingly, an electron would take nearly a century to traverse a distance of 45 Å. In bioenergetic membranes, large distances are traversed by introducing several cofactors into the membrane so as to shorten the distance between any two redox pairs. In photosynthetic systems, an electron traverses the width of the thylakoid membrane in less than 1 μs due to the presence of multiple electron transfer cofactors. With detailed knowledge of the factors that affect electron transfer in proteins, we now turn to how nature has incorporated these concepts into the design of a photosynthetic reaction center.

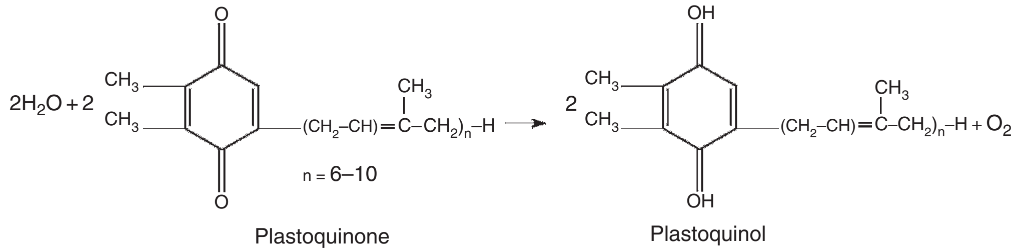
The Photosynthetic Reaction Center

The pigment–protein complex that is responsible for translocating the electron across the photosynthetic membrane is termed the photosynthetic reaction center. It comprises the antenna pigments, the organic and inorganic molecules that function as electron transfer cofactors, and the proteins that provide the scaffold for these components.

Oxygenic phototrophs employ two photosynthetic reaction centers in series for achieving transmembrane electron transfer.

Photosystem II

The net reaction performed by PS II can be summarized as:



[6]

The structural model of cyanobacterial PS II (**Figure 5**) shows that the redox cofactors bound to the D1 and D2 proteins form two branches that are arranged symmetrically along a pseudo-C_2 axis of symmetry (**Figure 6**).

The electron transfer chain in PS II starts at a special pair of chlorophyll *a* (Chl *a*) molecules termed P_{680}, named for its peak absorbance in the visible region. The magnesium atoms between the two Chl *a* molecules (P_{D1} and P_{D2}) are separated by a distance of only 7.6 Å. When the exciton reaches P_{680} via the antenna system, it becomes excited to the singlet state and the electron is transferred to the primary acceptor, a pheophytin molecule. A bridging Chl *a* molecule acts as an intermediate between P_{680} and the pheophytin. The bridging Chl *a* molecules on either side of the pseudo-C_2 axis (Chl_{D1} and Chl_{D2}) are located 10.4 and 10.3 Å, respectively, from P_{D1} and P_{D2}. The two pheophytin molecules (Pheo_{D1} and Pheo_{D2}) are located at a distance of 10.5 and 10.7 Å, respectively, from Chl_{D1} and Chl_{D2}.

The charge-separated state between $P_{680}{}^+$ and Pheo^- is stabilized by the transfer of the electron from Pheo^- to Q_A and then to Q_B, both of which are molecules of

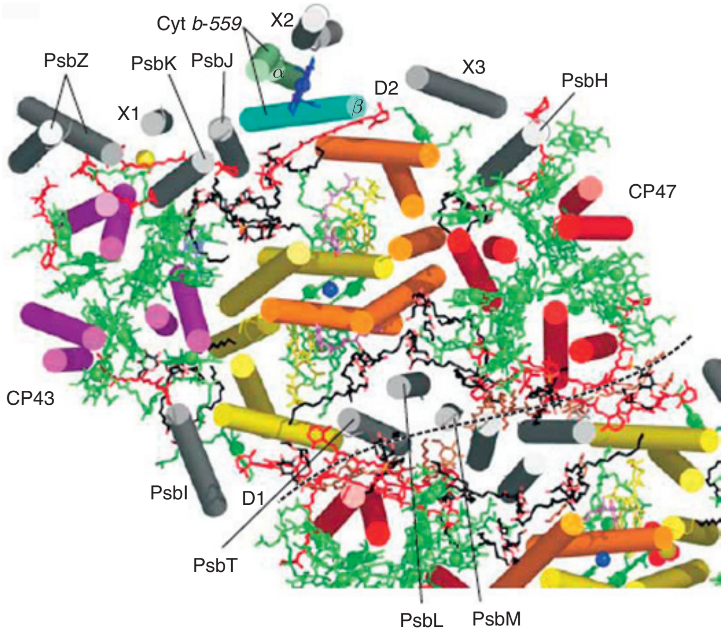

Cyt *b-559*
X2
PsbZ
PsbK
PsbJ
α
β
D2
X3
PsbH
X1
CP47
CP43
PsbI
D1
PsbT
PsbL
PsbM

Figure 5 Overall view of the PS II monomer. Transmembrane α-helices are represented by cylinders. The main subunits are colored as follows: reaction center subunits D1 (yellow) and D2 (orange), antenna subunits CP43 (magenta) and CP47 (red), and the α and β subunits of cytochrome *b559* (green and cyan, respectively). Low molecular mass subunits are colored gray. Cofactors are colored green (Chl *a*), yellow (pheophytin), red (carotenoid), blue (heme), violet (quinone), and black (lipids).

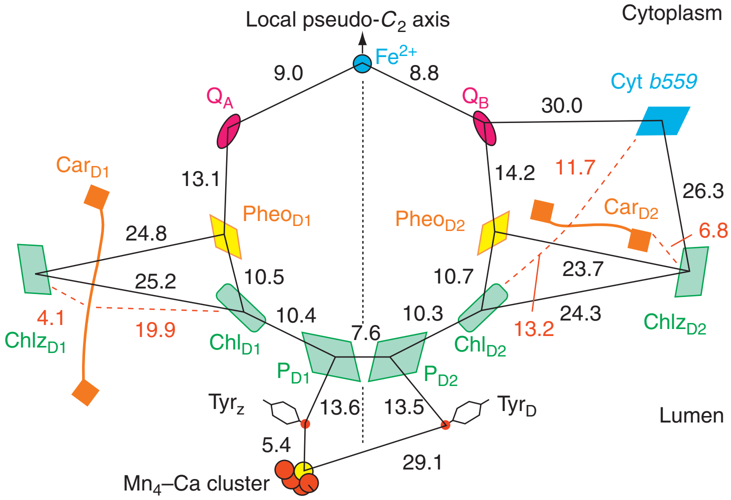

Local pseudo-C_2 axis
Cytoplasm
Fe^{2+}
9.0
8.8
Q_A
Q_B
30.0
Cyt *b559*
Car_{D1}
13.1
14.2
11.7
26.3
$Pheo_{D1}$
$Pheo_{D2}$
Car_{D2}
24.8
6.8
25.2
10.5
10.7
23.7
4.1
10.4
10.3
24.3
$Chlz_{D1}$
19.9
7.6
13.2
$Chlz_{D2}$
Chl_{D1}
Chl_{D2}
P_{D1}
P_{D2}
Tyr_Z
13.6
13.5
Tyr_D
Lumen
5.4
29.1
Mn_4–Ca cluster

Figure 6 View of redox-active cofactors and the electron transport chain of PS II along the membrane plane. The pseudo-C_2 axis is represented by the dotted line with the arrow. Selected distances (in angstrom) are drawn between cofactor centers (black lines) and edges of π-systems (red dotted lines).

plastoquinone. A nonheme iron (Fe^{2+}) located between Q_A and Q_B aids in the electron transfer. The Q_A site is 13 Å distant from $Pheo_{D1}$, while the Q_A and Q_B sites are separated by 18 Å, with the nonheme iron precisely in the middle. Because a quinone molecule (Q_B) acts as the terminal electron acceptor, PS II is classified as a Type II reaction center.

Water, through the action of the O_2-evolving complex, reduces oxidized P_{680} via Tyr_Z, thereby precluding charge recombination between $P_{680}{}^+$ and $Q_B{}^-$ and thus forming the $P_{680}Q_AQ_B{}^-$ charge-separated state. When a second exciton reaches P_{680}, the process is repeated, creating an unstable $P_{680}Q_A{}^-Q_B{}^-$ charge-separated state. Two protons are taken up from the medium, and the stable $P_{680}Q_A(Q_BH_2)$

state is generated. Plastoquinol (Q_BH_2) has a low affinity for the Q_B-binding site. It diffuses within the membrane to the cytochrome b_6f complex, which oxidizes plastoquinol to plastoquinone, and by virtue of the protonmotive Q-cycle translocates up to two protons per electron across the membrane. The regenerated plastoquinone diffuses back to the Q_B-binding site to participate in another round of light-induced electron transfer. The cytochrome b_6f complex passes the electron to a soluble carrier such as plastocyanin (a copper protein) or cytochrome c_6 (a heme protein), which diffuses laterally along the lumenal space, donating its electron eventually to PS I.

The 38 kDa D1 protein (also known as PsbA) and the 39 kDa D2 protein (also known as PsbD) contain all the electron transport cofactors of PS II. The 56 kDa CP47 protein (also known as PsbB) and the 50 kDa CP43 protein (also known as PsbC) harbor most of the antenna chlorophylls and carotenoids associated with PS II. The 9 kDa PsbE and the 4.5 kDa PsbF proteins constitute the α and the β subunits of cytochrome *b559*, which is present to prevent radical formation under conditions of suboptimal electron flow. PS II contains additional subunits (denoted PsbG–PsbT), many of which have poorly understood roles.

The kinetics of electron transfer between most of the redox cofactors have been determined to a high degree of precision (**Figure 7**). The electron is transferred from P_{680} to Pheo in 4 ps and then to Q_A in 200 ps. The $Q_A \rightarrow Q_B$ step requires about 100 and 200 µs when Q_B becomes singly and doubly reduced, respectively.

Even though any given reduced acceptor can recombine with the $P_{680}{}^+$ cation, forward electron transfer to the next acceptor is at least 1000 times faster than charge recombination. Thus, the quantum yield (i.e., the number of charge-separated events created divided by the number of photons absorbed) is extremely high.

The bioenergetics of electron transfer further reflect the artful design of the photosynthetic reaction center. All electron transfer steps on the acceptor side of PS II are thermodynamically downhill, that is, there is a loss in Gibbs free energy at every step that 'pays' for the successively longer charge time of charge separation.

Photosystem I

The net reaction performed by PS I can be summarized as:

$$2\mathrm{NADP}^+ + 2\mathrm{H}^+ + 4\,\mathrm{e}^- \rightarrow 2\mathrm{NADPH} \qquad [7]$$

PS I employs the same design principles as PS II to generate and stabilize a charge-separated state. The electron transfer chain starts at a special pair of chlorophyll *a* (Chl *a*) molecules termed P_{700}, named for its peak absorbance in the visible

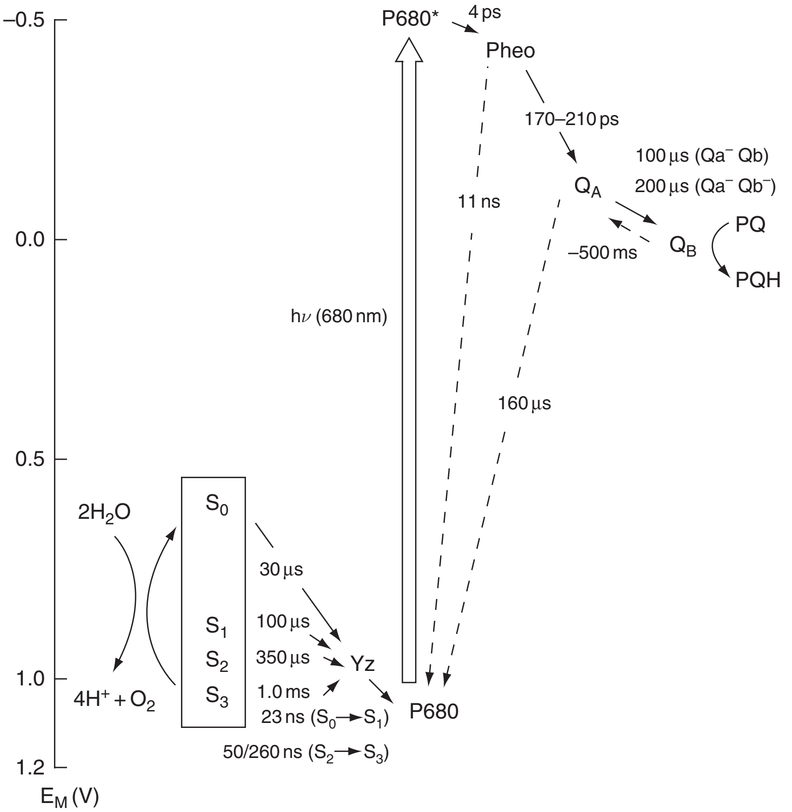


Figure 7 The components of the PS II reaction center depicted with redox potential on the *y*-axis and rate of electron transfer on the *x*-axis.

region. When P_{700} becomes excited to the singlet state, its electron is transferred to the primary acceptor, a Chl *a* monomer. An additional molecule of Chl *a* located between P_{700} and A_0 acts as an electron transfer bridge. The charge-separated state is stabilized by rapid transfer of the electron to a molecule of bound phylloquinone (A_1) and then to a series of [4Fe–4S] clusters termed F_X, F_A, and F_B, which function as an electron transfer wire. Because the terminal acceptors are iron–sulfur clusters, PS I is a Type I reaction center.

A model based on the 2.5 Å resolution crystal structure of the trimeric PS I reaction center from the cyanobacterium *Synechoccoccus elongatus* (PDB ID 1JB0) has provided detailed structural information on the arrangement of proteins and cofactors (**Figure 8**). Each monomer of the PS I heterodimer contains 96 Chl *a* molecules, 22 carotenoids, and 4 lipids. All of the organic cofactors in the electron transport chain are arranged in two pseudo-C_2 symmetric branches along the PsaA–PsaB heterodimer (**Figure 9**). However, unlike PS II, in which electron transfer occurs only along one branch, both the branches in PS I participate to varying degrees. In higher plants and algae, electron transfer along the PsaB branch is nearly as active as along the PsaA branch, whereas in cyanobacteria electron transfer is more asymmetrical, with only a small minority of electrons transported along the PsaB branch.

However, which branch is most active has little practical consequence, as the two branches converge at F_x, a unique interpolypeptide [4Fe-4S] cluster ligated by two cysteine residues from PsaA and two cysteine residues from PsaB. F_x is possibly the most reducing iron–sulfur cluster in biology, with a measured midpoint potential of −710 to −730 mV. The terminal [4Fe–4S] clusters, F_A and F_B, are located in the PsaC subunit, which is located on the stromal side of the membrane. To our knowledge, PsaC is the only membrane-associated protein whose three-dimensional structure has been solved in both the bound and unbound forms. PsaD and PsaE are two additional stromal proteins that flank PsaC, stabilizing its association with the heterodimeric core. These stromal proteins are vital for the docking of soluble electron acceptors such as ferredoxin and flavodoxin. Cyanobacterial PS I contains seven additional subunits (PsaF, PsaI, PsaJ, PsaK, PsaL, PsaM, and PsaX) whose roles are not well established. Some function as additional antenna proteins by binding one or two chlorophyll *a* molecules. Others, such as PsaL, along with PsaI and PsaM, contribute to the trimerization of cyanobacterial PS I. It is interesting that the absence of a portion of the C-terminus in the PsaL subunit leads to a completely monomeric PS I in higher plants and algae.

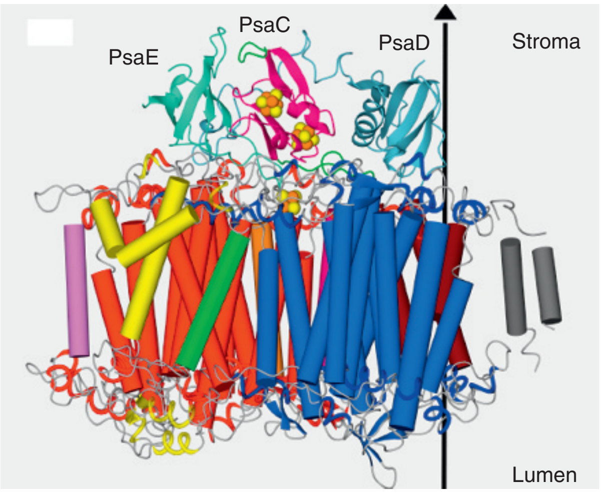


Figure 8 Side view of the arrangement of all proteins in one monomer of PS I. The main subunits are colored as follows: PsaA (blue), PsaB (red), PsaC (pink), PsaD (turquoise), PsaE (green), and PsaF (yellow). The iron–sulfur clusters in PsaC are colored yellow. The antenna pigments have been omitted for clarity. The vertical line (right) depicts the crystallographic C_3 axis.

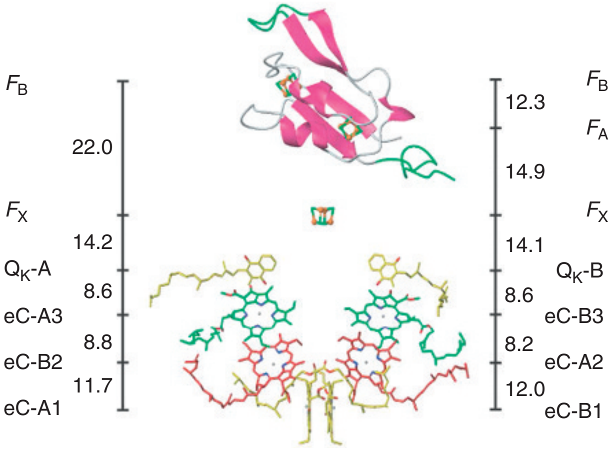


Figure 9 The arrangement of cofactors in PS I with center-to-center distances (in angstrom). The Chl *a* molecules, eC-A_1 and eC-B_1, constitute the special pair of chlorophylls P_{700}. The chlorophylls eC-A3 and eC-B3 represent A_0 on the PsaA and PsaB branches, respectively. Q_K-A and Q_K-B represent phylloquinone (A_1) on the PsaA and PsaB branches, respectively. The three [4Fe–4S] clusters are F_X, F_A, and F_B.

The electron transfer kinetics among the bound cofactors in PS I are roughly comparable to those in PS II (**Figure 10**). The primary electron donor, P_{700}, becomes oxidized in ∼20 ps, and the electron is transferred from A_0 to A_1 in 50 ps. The electron transfer step from A_1 to F_X is biphasic with lifetimes of ∼200 ns and 20 ns. It is likely that the slower kinetic phase represents electron transfer along the PsaA branch of cofactors, while the faster phase represents electron transfer along the PsaB branch. The rates of electron transfer between F_X, F_A, and F_B are not known with certainty; however, soluble ferredoxin is known to be reduced within 500 ns. The reduced ferredoxin interacts with the enzyme ferredoxin-$NADP^+$ oxidoreductase

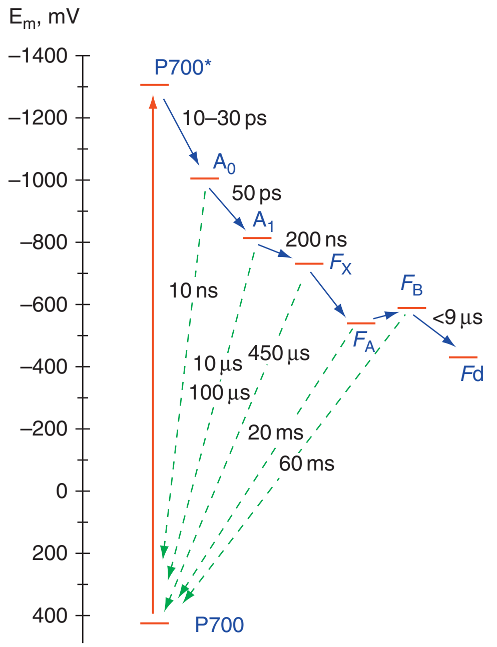


Figure 10 The components of the PS I reaction center depicted with redox potential on the *y*-axis and rate of electron transfer on the *x*-axis.

(FNR) to transfer its electron to NADP$^+$. NADPH is used along with the ATP generated from the transmembrane proton gradient to fix CO_2 into hexose sugars.

The extremely low midpoint potentials of A_1 and F_X are intriguing and make PS I an excellent model for studying the influence of the protein environment on redox cofactors in biological systems. Our current knowledge of this topic is largely restricted to theoretical predictions based on influences such as uncompensated charges and polarities of amino acid side groups, the fixed dipole moment of the peptide bond in the polypeptide chain, and solvent accessibility to the cofactors. A change in the protein environment introduced by mutagenesis will usually lead to a change in the redox potential of one or more electron transfer cofactors, which, in turn, results in an altered rate of electron transfer. The change in kinetics can be related to a change in midpoint potential using Marcus theory. As an example, the substitution of a negatively charged aspartate near the A_1 quinone in PS I with a neutral or positively charged residue results in a decrease in the rate of electron transfer to F_X, which implies a lower driving force between the two cofactors. Conversely, removal of a negative charge near the F_X cluster in PS I leads to higher rates of electron transfer from A_1 and hence a greater driving force between the two cofactors. The experimental data agree well with the electrostatic calculations based on the X-ray crystal structure of PS I.

Cyanobacterial PS I is an ideal candidate for these experimental studies, as it is relatively straightforward to determine the rates of electron transfer using time-resolved optical or EPR spectroscopy. The presence of a well-established transformation system facilitates the construction of site-directed variants. The availability of a 2.5 Å resolution X-ray crystal structure of PS I is an added advantage because it allows accurate predictions to be made, which can then be tested by experiments.

Although PS II and PS I use similar design principles in achieving charge separation, they have complementary roles in photosynthesis by functioning at the two extremes of the biological redox scale. PS II has evolved to generate a strong oxidant to split H_2O, whereas PS I has evolved to generate a strong reductant to produce NADPH.

With knowledge of how the electron from H_2O is transported across a thermodynamic gradient to produce NADPH, we now focus on the biochemistry of converting CO_2 into carbohydrate.

Calvin–Benson–Bassham Cycle

The Calvin–Benson–Bassham (CBB) cycle of carbon fixation can be divided into two stages. The first involves the trapping of CO_2 as a carboxylate and its subsequent reduction to glyceraldehyde-3-phosphate (G3P). The second involves the regeneration of the acceptor molecule for the next cycle of carbon fixation. A schematic view of the CBB cycle is depicted in **Figure 11**.

Atmospheric CO_2 diffuses into the stroma where it is added to the five-carbon acceptor molecule, ribulose-1,5-biphosphate (RuBP). The enzyme Rubisco, which is the most abundant protein on earth, catalyzes the reaction. The product is a six-carbon intermediate that is cleaved into two molecules of 3-phosphoglycerate (3PG). At this point, CO_2 has already been fixed into a carbohydrate. The remainder of the cycle is dedicated to the formation of hexose sugars and to the regeneration of RuBP.

Each molecule of 3PG is phosphorylated by the ATP-dependent enzyme phosphoglycerate kinase, liberating ADP in the process. The 1,3-biphosphoglycerate so formed is then reduced to G3P by NADPH, with the accompanying loss of a phosphate. This reaction is catalyzed by the enzyme G3P dehydrogenase. For each CO_2 molecule that passes through these steps, two molecules of ATP are hydrolyzed and two molecules of NADPH are oxidized. Every new molecule of hexose requires six CO_2 molecules to enter the cycle. That requires the formation of 12 G3P and therefore 12 ATP and 12 NADPH are required.

The conversion of G3P into carbohydrates is a complex multistep process involving several enzymes. G3P can be isomerized to dihydroxyacetone phosphate (DHAP) by triose phosphate isomerase. Thus, the 12 G3P molecules essentially form an interconvertible pool of G3P and DHAP. At this stage, the pathway bifurcates toward two goals: the production of hexoses and the regeneration of RuBP. Six molecules of G3P (4 G3P plus 2 DHAP) are

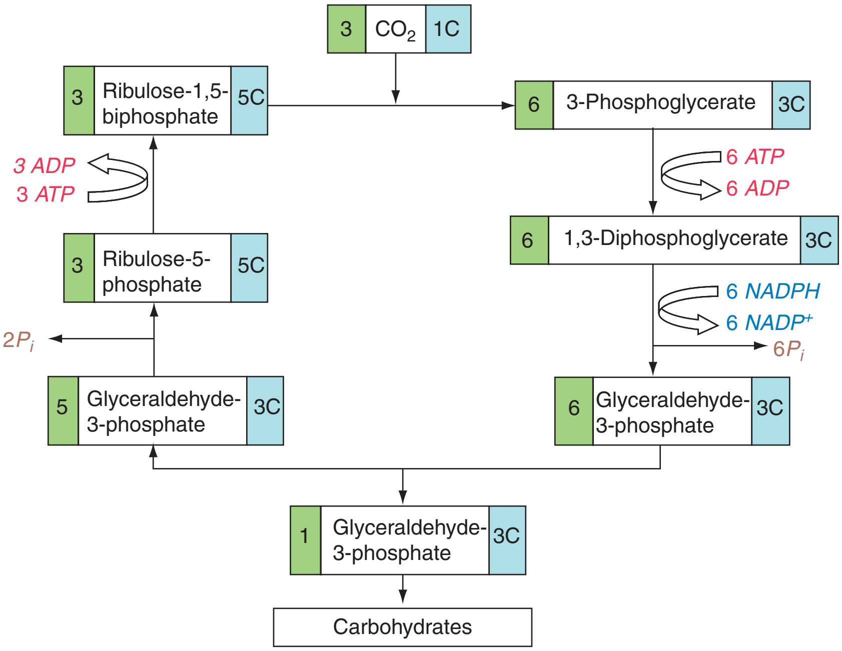


Figure 11 Schematic view of the Calvin–Benson–Bassham (CCB) cycle of carbon fixation. The net uptake of ATP and NADPH at each step is indicated.

diverted to the regeneration pathway, while the remaining six molecules (3 G3P plus 3 DHAP) are used for carbohydrate synthesis.

Three molecules of G3P combine with three molecules of DHAP via the enzyme fructose biphosphate aldolase to yield three molecules of fructose-1,6-biphosphate (FBP). The FBP is then dephosphorylated to provide three molecules of fructose-6-phosphate (F6P).

Of these, two molecules will be used in the regeneration pathway, leaving one as the net product of the CCB cycle. F6P is isomerized to glucose-6-phosphate (G6P) and finally to glucose-1-phosphate (G1P), which is the precursor for oligosaccharide and polysaccharide formation. G1P can be hydrolyzed to form glucose, or it can be converted to amylose and sucrose via separate pathways.

The input molecules for the regeneration cycle are 4 G3P, 2 DHAP, and 2 F6P molecules. Enzymes termed *trans*-ketolases and aldolases perform molecular rearrangements that are necessary to form five-carbon molecules from six-carbon and three-carbon molecules. The final step in the regeneration of RuBP is a phosphorylation reaction catalyzed by the ATP-dependent enzyme ribulose-5-phosphate kinase. An additional six ATP are required for six rounds of this step. The overall CCB cycle can thus be summarized as:

$$6CO_2 + 18ATP + 12NADPH + 12H_2O \rightarrow C_6H_{12}O_6 + 18ADP + 18P_i + 12NADP^+ + 6H^+ \quad [8]$$

Anoxygenic Photosynthesis

As the name suggests, anoxygenic photosynthetic bacteria do not evolve O_2 as a by-product of photosynthesis. These descendants of ancient microbes contain only one type of reaction center and hence the electrons used to reduce CO_2 are taken from highly reduced molecules such as succinate and sulfide. Although most photosynthetic bacteria use the CCB cycle to fix carbon, some are able to fix atmospheric CO_2 by other biochemical pathways. Most anaerobic phototrophs can survive only under very low concentrations of O_2.

Despite these differences, the general principles of energy transduction in anoxygenic photosynthesis are similar to those in oxygenic photosynthesis. The primary chromophore belongs to a family of molecules called bacteriochlorophylls. There are six types of bacteriochlorophylls, denoted bacteriochlorophyll (BChl) *a*, *b*, *c*, *d*, *e*, and *g* (**Figure 12**). They are similar to chlorophylls, but absorb light in the near-infrared region (**Figure 13**). As in aerobic photosynthesis, electron transfer is coupled to the generation of a proton gradient that is used to synthesize ATP. The energy required to reduce CO_2 is provided by ATP and NADH, a molecule similar to NADPH but lacking the phosphate.

Purple Bacteria

Purple photosynthetic bacteria are a versatile group of proteobacteria that can be classified further into purple

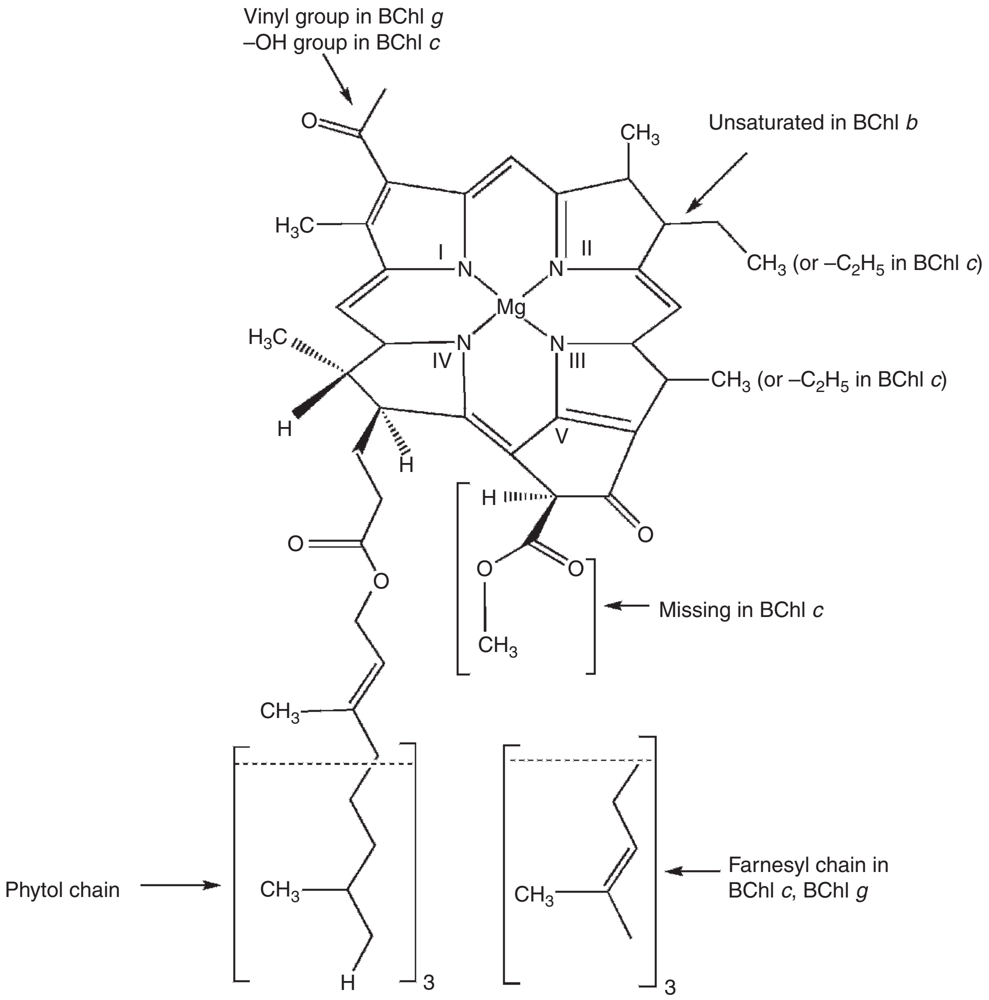

Vinyl group in BChl *g*
–OH group in BChl *c*
O
CH_3
Unsaturated in BChl *b*
H_3C
I
II
N
N
Mg
CH_3 (or $-C_2H_5$ in BChl *c*)
H_3C
N
N
IV
III
CH_3 (or $-C_2H_5$ in BChl *c*)
H
H
V
H
O
O
O
O
O
Missing in BChl *c*
CH_3
CH_3
Phytol chain
CH_3
H
3
CH_3
3
Farnesyl chain in BChl *c*, BChl *g*

Figure 12 Chemical structure of BChl *a*, one of the pigments employed by anoxygenic phototrophs. The structural changes resulting in other forms of bacteriochlorophyll are also indicated.

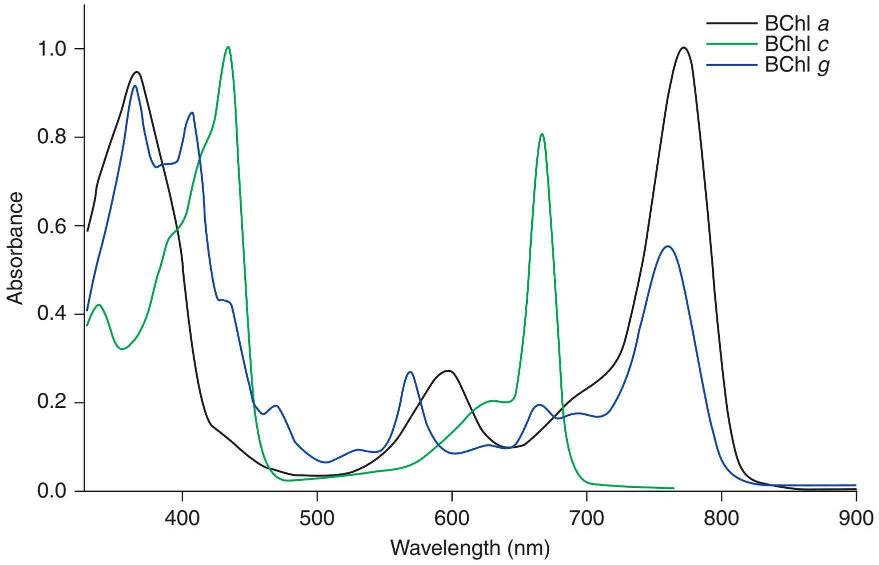


Figure 13 Absorption spectrum of BChl *a*, BChl *c*, and BChl *g* in solution.

nonsulfur bacteria and purple sulfur bacteria. All purple bacteria use a Type II reaction center to generate a proton gradient for ATP synthesis, that is, there is no formation of NADPH. The reductant for carbon fixation is derived from organic compounds such as succinate and malate (nonsulfur bacteria) or from inorganic sulfide (sulfur bacteria). Light-driven electron transfer in purple bacteria is cyclic and hence no net oxidation or reduction occurs.

Purple nonsulfur bacteria are found in ponds, mud, and sewage. Purple sulfur bacteria are obligate anaerobes and are found in illuminated anoxic zones of lakes where H_2S accumulates and also in geothermal sulfur springs. Both fix carbon via the CBB cycle.

All purple bacteria have a very efficient antenna system consisting of BChl *a*, BChl *b*, and carotenoids. The presence of purple carotenoids such as spirilloxanthin gives these bacteria their distinct color. The first three-dimensional X-ray crystal structures of a photosynthetic reaction center were from purple nonsulfur bacteria (*Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*). The basic composition of their reaction centers is similar to that of PS II. The primary donor is a special pair of BChl *a* molecules, which, after excitation by light, transfer the electron to bacteriopheophytin, the primary electron acceptor. The charge-separated state is stabilized by successive electron transfer to two ubiquinone molecules, Q_A and Q_B. After two cycles of reduction, two protons are taken up from inside the membrane to form the doubly reduced dihydroubiquinol in the Q_B site. Dihydroubiquinol diffuses to the cytochrome *bc₁* complex, where it becomes oxidized, regenerating ubiquinone. The cytochrome bc_1 complex employs the protonmotive Q-cycle and translocates up to two protons per electron across the membrane. The energy stored in the resulting electrochemical proton gradient is used to synthesize ATP via the membrane-bound ATP synthase complex. The cytochrome bc_1 complex completes the cycle by transferring the electron back to the primary donor via the soluble carrier protein cytochrome *c*.

Green Sulfur Bacteria

Green sulfur bacteria such as *Chlorobium tepidum* and *Chlorobium vibrioforme* belong to the phyla Chlorobi and are strictly anaerobic photoautotrophs. They use reduced sulfur compounds as their electron donors and fix carbon using the reverse TCA cycle. Unlike purple bacteria, light-induced electron transfer is noncyclic in green sulfur bacteria; hence NADPH is generated. These bacteria live in sulfur-rich environments that have characteristically low light intensities. They employ a unique antenna complex termed the chlorosome, which comprises BChl *c*, BChl *d*, and BChl *e*. It is the largest known antenna structure in biology, with each chlorosome containing ~200 000 BChl molecules. The habitats of green sulfur bacteria necessitate such an extensive antenna system, requiring a very large optical cross section to capture the few available photons. The light energy is transferred to a homodimeric Type I reaction center via the BChl *a* containing the Fenna–Matthews–Olsen (FMO) protein. The FMO protein is soluble in water, and was the first chlorophyll-containing protein to have its three-dimensional structure solved. The reaction center core is a homodimer of PscA, and it contains most of the redox cofactors. Electron transfer begins at P_{840}, a special pair of BChl *a* molecules, and proceeds through the primary acceptor, a Chl *a* molecule monomer, and three [4Fe–4S] clusters F_X, F_A, and F_B. It is uncertain whether a quinone functions as an intermediate electron transfer cofactor between A_0 and F_X. F_A and F_B are bound to a protein named PscB, which has an unusually long N-terminal segment of proline, lysine, and arginine residues. A protein named PscD is thought to be involved in the docking of soluble ferredoxin and in the stabilization of the FMO protein. Another protein, PscC, is a tightly bound cytochrome, c_{551}, that donates electrons to P_{840}.

Heliobacteria

Heliobacteria (e.g., *Heliobacterium mobilis* and *Heliobacterium modesticaldum*) are members of the phylum Firmicutes and are the only known Gram-positive photosynthetic organisms. They were discovered 25 years ago in soil on the campus of Indiana University, Bloomington. Heliobacteria are anaerobic photoheterotrophs that fix nitrogen and are commonly found in rice fields. They can grow on selected organic substrates like pyruvate, lactate, and butyrate. Heliobacteria do not contain ribulose-1,5-bisphosphate or ATP-citrate lyase, the two enzymes commonly used in carbon fixation, but rather incorporate carbon via an incomplete reductive carboxylic acid pathway. These bacteria use BChl *g* as their primary pigment and employ a simple homodimeric Type I reaction center to perform noncyclic electron transfer. The components of the electron transfer chain are similar to green sulfur bacteria except that the pigment used as the special pair (P_{798}) is BChl *g*. The reaction center core is a homodimer of PshA, and it contains the primary donor and acceptor chlorophylls and the F_X iron–sulfur cluster. The F_A and F_B iron–sulfur clusters are harbored on a low molecular mass polypeptide termed PshB. Similar to the reaction centers in the phylum Chlorobi, the participation of a quinone as an electron transfer cofactor between A_0 and F_X is still under debate.

Little or no structural information is available on any homodimeric Type I reaction center. Based on analogy with PS I, it is believed that a bifurcating electron transfer chain with two equivalent branches of cofactors exists in these reaction centers, but there is no spectroscopic evidence yet to support this proposal.

Other Photosynthetic Bacteria

Some species of photosynthetic bacteria do not fall under any of the previously discussed categories. The green gliding bacteria (*Chloroflexi*), also known as green filamentous bacteria, can grow photosynthetically under anaerobic conditions or in the dark by respiration under aerobic conditions. Like green sulfur bacteria, they harvest light by using chlorosomes, but like purple bacteria, they employ a Type II reaction center. These poorly studied organisms fix CO_2 via the 3-hydroxypropionate pathway.

The most recent addition to the list of photosynthetic microbes is an acidobacterium, *C. thermophilum*, which reportedly synthesizes BChl *a* and BChl *c* in aerobic environments. This organism was isolated from microbial mats at an alkaline hot spring and is thought to contain chlorosomes and a homodimeric Type I reaction center. Further studies are needed to determine whether the photosynthetic apparatus has new and interesting features, or whether it falls into a typical Type I class.

The Evolution of Photosynthesis

The origin of photosynthesis is such an ancient event that the details of how this biological process developed may be irretrievably lost. Nevertheless, an extensive interdisciplinary effort involving researchers in biochemistry, genetics, biophysics, geology, biogeochemistry, and bioinformatics has led to new and important insights into the evolutionary history of photosynthesis.

There is widespread agreement that the first photosynthetic organisms were anoxygenic prokaryotes and that the cyanobacteria, with their much more complicated system of two linked photosystems, were a later development. However, the origin of the most primitive photosynthetic machinery remains shrouded in mystery.

According to one school of thought, a common single polypeptide could have been the ancestor to both Type II and Type I reaction centers. This primordial reaction center would have diverged into a homodimeric reaction center and subsequent development would have led to the present reaction centers found in anoxygenic phototrophs. Alternately, the formation of a homodimeric reaction center might have been the initial photosynthetic event (**Figure 14**). From a geometrical perspective, a three-dimensional surface is easiest to construct by abutting two two-dimensional surfaces. A highly protected environment at the interface of the two identical proteins would neatly solve the problem of shielding the electron transfer cofactors from water without requiring a highly folded three-dimensional structure such as the interior of a protein. Inherent in both ideas, the formation of a heterodimeric core would have been a result of gene divergence from a homodimeric core. The similar motifs of charge separation and charge stabilization in both Type I and Type II reaction centers are certainly compatible with a common origin. Genetic fusion between two organisms, one having a Type II and the other a Type I reaction center, would have led to the colocation of both in the same organism. This fusion organism likely evolved into the present-day cyanobacteria.

The terminal electron acceptor of the primordial reaction center in either case could have been a quinone or an iron–sulfur cluster. The recruitment of a bacterial ferredoxin later in evolution would account for the presence of

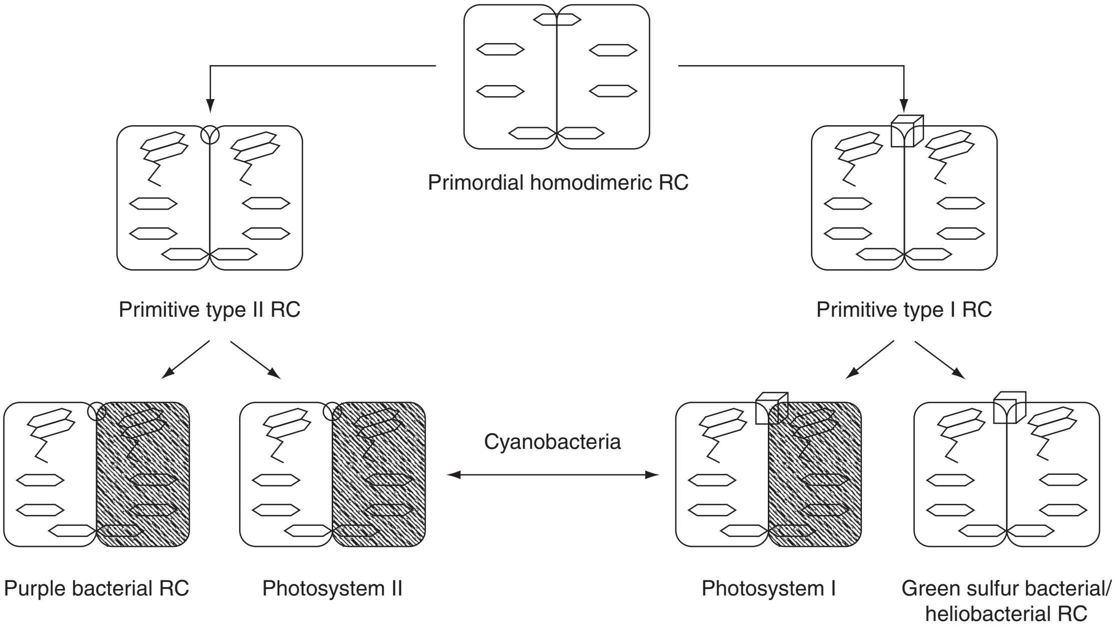


Figure 14 Schematic representation of the homodimeric 'primordial reaction center' model of photosynthetic evolution.

F_A and F_B in heterodimeric as well as homodimeric Type I reaction centers. It is also possible that a completely different moiety could have functioned as the primordial terminal acceptor, with subsequent modifications that led to the emergence of Type II and Type I reaction centers.

In Type II reaction centers, the transition from homodimeric to heterodimeric state might have occurred to specialize the function of the Q_A and Q_B quinones as one-electron and two-electron gates, respectively. The reason for the transition is less clear in Type I reaction centers, which do not require a division of labor between the two quinones. It is nevertheless interesting that heterodimeric Type I reaction centers are exclusively associated with cyanobacteria, algae and plants, organisms that employ accessory antenna chlorophyll proteins. They might have evolved from a homodimeric to a heterodimeric state to provide specialized binding sites for these additional structures. The two branches of redox cofactors are highly symmetrical in heterodimeric Type I and Type II reaction centers, suggesting that this transition has not resulted in a significant alteration of the protein environment surrounding the electron transport cofactors.

Although analysis of protein similarity is a highly effective method to predict evolutionary events, it does not help in understanding the development of complex pigment molecules such as chlorophyll. The original Granick hypothesis holds that biosynthetic pathways for the formation of chlorophyll recapitulate their evolution. It proposes that the pathway is built forward, with each step fulfilling a function, eventually being replaced by the next step selected for improved utility. The problem with this proposal is that the synthesis of bacteriochlorophyll proceeds through a chlorophyll-like intermediate step. A strict interpretation of the Granick hypothesis would therefore imply that bacteriochlorophyll-containing organisms (anoxygenic) evolved later than chlorophyll-containing organisms (oxygenic). Because the opposite is most likely the case, the reaction centers and chlorophylls may have followed a dissimilar history in evolution.

The origin of the water-splitting complex is also uncertain, although some groups have postulated that it evolved from a manganese-containing catalase. According to this idea, weak electron donors such as H_2O_2 and $Fe(OH)^+$ once provided the electrons to PS II. The incorporation of the water-splitting complex might have been driven by the necessity to replace these rare electron donors with the most abundant electron donor on earth, H_2O.

Future Research Directions

Although extensive research in the last four decades has led to a good understanding of the overall design philosophy of photosynthetic electron transfer, there remain many unanswered questions. The mechanism behind the splitting of H_2O is still unknown, primarily because the S_4 state of the manganese cluster, which is the starting point for O–O bond formation, has not been observed. High-resolution crystal structures are invaluable, but they only provide a static ground-state depiction of the proteins and cofactors. Sophisticated spectroscopic techniques, particularly electron paramagnetic resonance, are required to probe the excited states, providing precise details of the critical steps in oxygenic photosynthesis.

Homodimeric Type I reaction centers remain poorly understood. The presence of two branches of electron transfer cofactors in identical environments begs the question of how charge separation is initiated and how the electron ‘chooses’ which of two equivalent pathways to take. The main hindrance to progress lies in the inability to spectroscopically distinguish electron transfer on either branch.

Photosynthetic reaction centers are undoubtedly the best model system for studying the influence of the protein environment on the redox potentials of organic and inorganic cofactors. This is best exemplified by comparing the quinones in Type II and Type I reaction centers, which are structurally similar but differ in redox potential by hundreds of millivolts. Q_A and Q_B, which are both plastoquinones, have midpoint potentials of $-150\,\text{mV}$ and $+100\,\text{mV}$, respectively, when bound to PS II. An even more dramatic case is provided by A_1, the phylloquinone bound to PS I. Its redox potential in organic solvent is similar to that of plastoquinone, but when bound to PS I, it has a midpoint of $-800\,\text{mV}$. Thus, the redox cofactors are tuned by the protein to provide an appropriate midpoint potential for a given electron transfer step. Detailed knowledge of how the tuning occurs will be exceedingly useful in the designing of artificial photosynthetic systems.

Indeed, the next decade will probably see a major advance in artificial photosynthesis, mainly due to the concern over the need to develop new sources of energy after the depletion of fossil fuels. The basic outline of solar energy conversion is now known from the knowledge of natural photosynthesis, and these principles can be used to design artificial organic and inorganic systems of high efficiency. A solar cell consisting of photosynthetic reaction centers from spinach and from purple bacteria layered on a silver electrode has already been shown to generate an electric current. Efforts to develop synthetic counterparts of the reaction center and the oxygen-evolving complex have had limited success due to the inability to replicate the complex environment of a living cell. However, the coming years will undoubtedly see new developments in this field.

Several research groups are also trying to use photosynthetic organisms to generate H_2 that can be used as an alternate source of stored solar energy. Several promising ideas include the manipulation of cyanobacterial genes to enhance H_2 production in whole cells and the synthesis of a PS I–hydrogenase hybrid complex.

It is somewhat ironic that after depleting the fossil fuels that were produced by photosynthetic organisms in the first place we are again looking to photosynthesis for our long-term energy needs. Photosynthesis truly has come full circle.

Acknowledgments

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Pili, Fimbriae

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Further Reading

Glossary

curli A class of thin, irregular, and highly aggregated adhesive surface fibers expressed by *Escherichia coli* and *Salmonella* spp.

periplasmic chaperones A class of proteins localized within the periplasm of Gram-negative bacteria that facilitates the folding and assembly of pilus subunits, but which are not components of the final pilus structure.

phase variation Reversible on and off switching of a bacterial phenotype, such as pilus expression.

pilin Individual protein subunit of a pilus organelle (also known as a fimbrin). Immature pilins, containing leader signal sequences that direct the transport of pilins across the inner membrane of Gram-negative bacteria, are called propilins or prepilins.

sec *secretion system* A system involving at least seven proteins that mediates translocation of proteins across biological membranes. Proteins are targeted for *sec*-dependent secretion by the presence of an amino acid signal sequence.

sortase A transpeptidase that 'sorts' proteins containing the motif LPXTG into the cell wall fraction of Gram-positive bacteria by attachment to peptidoglycan.

type II secretion system A system of protein secretion in which the substrates are already-folded proteins. Type II secretion systems share many features with type IV pilus assembly.

type III secretion system A *sec*-independent secretion pathway, also known as T3SS. Numerous Gram-negative pathogens utilize T3SS to secrete and inject effector molecules into the cytosol of host eukaryotic cells.

usher Oligomeric outer-membrane proteins that serve as assembly platforms for some types of pili. Usher proteins can also form channels through which nascent pili are extruded from bacteria.

Abbreviations

AAF	aggregative adherence fimbria
BfpA	bundle-forming pilus
CAP	catabolite activator protein
CFA	colonization factor I
CS1	coli surface antigen 1
CTXΦ	cholera toxin phage
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
IncF1	F1 incompatibility
Lrp	leucine-responsive regulatory protein
MR	mannose-resistant
MS	mannose-sensitive
T3SS	type III secretion system
T4SS	type IV secretion systems
TCP	toxin coregulated pili
TcpA	toxin coregulated pilus

Defining Statement

Pili, also known as fimbriae, are proteinaceous, filamentous polymeric organelles expressed on the surface of bacteria. They range from a few fractions of a micrometer to $>20\,\mu\text{m}$ in length and vary from <2 to 11 nm in diameter. Their functions include mediation of cell-to-cell interactions, motility, and DNA uptake.

Pili are composed of single or multiple types of protein subunits, called pilins or fimbrins, which are typically arranged in a helical fashion. Pilus architecture varies from thin, twisting thread-like fibers to thick, rigid rods with small axial holes. Thin pili with diameters of 2–3 nm, such as K88 and K99 pili, are sometimes referred to as 'fibrillae'. Even thinner fibers (<2 nm), which tend to coil up into a fuzzy adhesive mass on the bacterial surface, are referred to as thin aggregative pili or curli. High-resolution electron microscopy of P, type 1, and S pili of *Escherichia coli*, and *Haemophilus influenzae* pili has revealed that these structures are composite fibers, consisting of a thick pilus rod attached to a thin, short distally located tip fibrillum. Pili are often expressed peritrichously around individual bacteria, but some, such as type IV pili, can be localized to one pole of the bacterium.

Pili are produced by both Gram-negative and Gram-positive bacteria. The numerous types of pili have been ascribed diverse functions in the adaptation, survival, and spread of both pathogenic and commensal bacteria. Pili can act as receptors for bacteriophage, facilitate DNA uptake and transfer (conjugation), and, in at least type IV pili, function in cellular motility. The primary function of most pili, however, is to act as scaffolding for the presentation of specific adhesive moieties. Adhesive pilus subunits (adhesins) are often incorporated as minor components into the tips of pili, but major structural subunits can also function as adhesins. Adhesins can mediate the interaction of bacteria with each other, with inanimate surfaces, and with tissues and cells in susceptible host organisms. The colonization of host tissues by bacterial pathogens typically depends on a stereochemical fit between an adhesin and complementary receptor architecture. Interactions mediated by adhesive pili can facilitate the formation of bacterial communities such as biofilms and are often critical to the successful colonization of host organisms by both commensal and pathogenic bacteria.

Historical Perspective and Classification of Pili

Pili were first noted in early electron microscopic investigations as nonflagellar, filamentous appendages of bacteria. In 1955, Duguid designated these appendages 'fimbriae' (plural, from Latin for thread or fiber) and correlated their presence with the ability of *E. coli* to agglutinate red blood cells. Ten years later Brinton introduced the term 'pilus' (singular, from Latin for hair) to describe the fibrous structures (F pili) associated with the conjugative transfer of genetic material between bacteria. Since then 'pilus' has become a generic term used to describe all types of nonflagellar filamentous appendages, and it is used interchangeably with the term 'fimbria'.

Historically, pili have been named and grouped based on phenotypic traits, such as adhesive and antigenic properties, distribution among bacterial strains, and microscopic characterizations. In the pioneering work of Duguid and collegaues, pili expressed by different *E. coli* strains were distinguished on the basis of their ability to bind to and agglutinate red blood cells (hemagglutination) in a mannose-sensitive (MS) versus a mannose-resistant (MR) fashion. Pili mediating MS hemagglutination by *E. coli* were designated type 1, and these pili have since been shown to recognize mannose-containing glycoprotein receptors on host eukaryotic cells. Morphologically and functionally homologous type 1 are expressed by many different species of Enterobacteriaceae. Despite their similarities, however, type 1 expressed by the various members of the Enterobacteriaceae family are often antigenically and genetically divergent within their major structural subunits. In contrast to type 1, most other pili so far identified either are nonhemagglutinating or mediate MR hemagglutination. These pili are very diverse and possess a myriad of architectures and different receptor-binding specificities and functions.

Pili were first identified on Gram-positive bacteria in 1968 by electron microscopy of *Corynebacterium renale*. Other *Corynebacterium* spp., and a number of other Gram-positive bacteria that live in the human oral cavity, were also subsequently shown to express pili. In part because of limitations on molecular genetic analysis in Gram-positive bacteria, relatively little has been known about these structures until recently. Since 2005, it has been discovered that three clinically significant Gram-positive pathogens, *Streptococcus pneumoniae*, group A *Streptococcus*, and group B *Streptococcus*, all express pili. Because pili hold promise as vaccine candidates, we can expect an increase in research on pili in Gram-positive bacteria in coming years.

In the more than 50 years since the discovery and initial characterization of pili, substantial advances have been made in our understanding of the genetics, biochemistry, and structural and functional aspects of these organelles. A vast number of distinct pilus structures have been described and new types of pili continue to be identified. Pili are now known to be encoded by virtually all Gram-negative organisms and are some of the best-characterized colonization and virulence factors in bacteria. In this article, we have classified pili expressed by Gram-negative bacteria into six different groups according to the mechanisms by which they are assembled; pili expressed by Gram-positive bacteria are addressed in a separate section. This classification scheme is not all-inclusive, but provides a convenient means for discussing the diverse types of pili, their functions, structures, and assembly. Representatives of various pilus types assembled by the different pathways discussed in the following sections are listed in **Table 1**.

Chaperone/Usher Pathway

All pilins destined for assembly on the surface of Gram-negative bacteria must be translocated across the inner membrane, through the periplasm, and across the outer

Table 1 Pilus assembly pathways

Assembly pathway	*Structure*	*Assembly gene products*^{a}	*Organism*	*Disease(s) associated with pilus expression*
Chaperone–usher pathway				
Thick, rigid pili	P pili	PapD/PapC	*E. coli*	Pyelonephritis/cystitis
	Prs pili	PrsD/PrsC	*E. coli*	Cystitis
	Type 1	FimC/FimD	*E. coli*	Cystitis
			Salmonella spp.	
			K. pneumoniae	
			X. fastidiosa	Plant diseases
	S pili	SfaE/SfaF	*E. coli*	UTI Newborn meningitis
	F1C pili	FocC/FocD	*E. coli*	Cystitis
	Hif pili	HifB/HifC	*H. influenzae*	Otitis media meningitis
	Haf pili	HafB/HafC	*H. influenzae Biogroup aegyptius*	Brazilian Purpuric Fever
	Types II and III pili	FimB/FimC	*B. pertussis*	Whooping cough
	MR/P pili	MrpD/MrpC	*P. mirabilis*	Nosocomial UTI
	PMF pili	PmfC/PmfD	*P. mirabilis*	Nosocomial UTI
	Long polar fimbriae	LpfB/LpfC	*S. enterica* ser. Typhimurium	Gastroenteritis
	Long polar fimbriae	LpfB/LpfC	*E. coli*	Diarrhea
	Pef pili	PefD/PefC	*S. enterica* ser. Typhimurium	Gastroenteritis
	Ambient-temperature fimbriae	AftB/AftC	*P. mirabilis*	UTI
	987P fimbriae	FasB/FasD	*E. coli*	Diarrhea in piglets
	REPEC fimbriae	RalE/RalD	*E. coli*	Diarrhea in rabbits
	AF/R1	AfrC/AfrB	*E. coli*	Diarrhea in rabbits
Thin, flexible pili	K99 pili	FaeE/FaeD	*E. coli*	Neonatal diarrhea in calves, lambs, piglets
	K88 pili	FanE/FanD	*E. coli*	Neonatal diarrhea in piglets
	F17 pili	F17D/F17papC	*E. coli*	Diarrhea
	F18 pili	FedC/ FedB	*E. coli*	Diarrhea in piglets
	MR/K pili	MrkB/MrkC	*K. pneumoniae*	Pneumonia
	Acu pili	AcuD/ AcuC	*Acinetobacter* spp. strain *BD413*	Opportunistic infections
Atypical structures	CS31A capsule-like protein	ClpE/ClpD	*E. coli*	Diarrhea
	Antigen CS6	CssC/CssD	*E. coli*	Diarrhea
	Myf fimbriae	MyfB/MyfC	*Y. enterolitica*	Enterocolitis
	PH 6 antigen	PsaB/PsaC	*Y. pestis*	Plague
	CS3 pili	CS3-1/CS3-2	*E. coli*	Diarrhea
	Envelope antigen F1	Caf1M/Caf1A	*Y. pestis*	Plague

(*Continued*)

Table 1 (Continued)

Assembly pathway	*Structure*	*Assembly gene products*[a]	*Organism*	*Disease(s) associated with pilus expression*
	Nonfimbrial adhesins I	NfaE/NfaC	*E. coli*	UTI newborn meningitis
	SEF14 fimbriae	SefB/SefC	*S. enteritidis*	Gastroenteritis
	Agregative adherence fimbriae I	AggD/AggC	*E. coli*	Diarrhea
	AFA-III	AfaB/AfaC	*E. coli*	Pyelonephritis
Alternate chaperone pathway	CS1	CooB/CooC	*E. coli*	Diarrhea
	CS2 pili	CotB/CotC	*E. coli*	Diarrhea
	CS4 pili		*E. coli*	Diarrhea
	CS5 pili	CsfB/ CsfC	*E. coli*	Diarrhea
	CS14 pili		*E. coli*	Diarrhea
	CS17 pili		*E. coli*	Diarrhea
	CS19 pili		*E. coli*	Diarrhea
	CFA/I pili	CfaA/CfaC	*E. coli*	Diarrhea
	Cable type II pili	CblB/CblC	*B. cepacia*	Opportunistic in cystic fibrosis patients
	Typhi colonization factor (Tcf)	SafB/SafC	*S. enterica* ser. Typhi	Typhoid
Type II secretion pathway	Type IVa pili	General secretion apparatus (main terminal branch) 14 to >20 proteins	*Neisseria* spp.	Gonorrhea, meningtitis
			P. aeruginosa	Opportunistic pathogen
			Moraxella spp.	Conjuntivitis, respiratory infections
			D. nodosus	Ovine footrot
			F. tularensis	Tularemia
			E. corrodens	Opportunistic pathogen
			L. pneumophila	Legionnaires' disease
			M. xanthus	Saprophyte
	Type IVa pili		*M. bovis*	Tuberculosis in cattle
			Azoarcus spp.	Denitrification and toluene metabolism
			B. bacteriovorus	Bacterial pathogen
			X. fastidiosa	Plant diseases
			V. parahaemolyticus	Gastroenteritis
			V. vulnificus	Gastroenteritis
	Type IVb pili	General secretion apparatus (Main terminal branch) 14 to >20proteins	*E. coli*	Diarrhea
	Bundle forming pili		*E. coli*	Diarrhea
	longus		*E. coli*	Diarrhea
	CFA/III		*E. coli*	
	R64 and ColIb-P9 pili			

	Toxin coregulated pili colonization factor (CfcA)		*V. cholera* *C. rodentium*	Cholera Murine colonic hyperplasia
	PilS pili		*S. enterica* ser. Typhi	Typhoid
Conjugative pilus assembly (Type IV secretion) pathway	F pili (IncF1)	Type IV export apparatus, 12 to 16 proteins	*E. coli*	Antibiotic resistance
	IncN, IncP, IncW-encoded pili		*E. coli* *A. tumefaciens*	 Crown gall disease
	T pili		*R. felis*	Rickettsiosis
Extracellular Nucleation/precipitation pathway	Curli	CsgG/CsgE/CsgF	*E. coli*	Sepsis
	Tafi (thin aggregative fimbrae)	AgfG/AgfE/AgfF	*S. enterica* ser. Typhimurium	Gastroenteritis
Type III secretion pathway	EspA pilus-like structures	Type III secretion apparatus, ∼20 proteins	*E. coli*	Diarrhea
	Hrp pili		*P. syringae*	Hypersensitive response (in resistant plants)
	Hrp pili		*E. amylovora*	Hypersensitive response
			X. campestris	Hypersensitive response
			R. solanacearum	Hypersensitive response
			Rhizobium spp.	Symbionts of legumes
			S. fredii	Symbionts of legumes
Pili in Gram-positive bacteria				
Sortases catalyzing transpeptidase reactions	SpaA-like pili	Sortases and pilins (SpaA, SpaB, SpaC, SpaD and SpaH)	*S. gordonii*	Dental plaque formation/ Endocarditis
			S. oralis	Dental plaque formation/ Endocarditis
			Corynebacterium spp.	Diphtheria/Oral pathogens
			Streptococcus spp.	Numerous diseases
			Actinomyces spp.	
Unknown			*A. photogonimos* *R. albus*	Rumen bacteria
	M. tuberculosis pili (MTP)		*M. tuberculosis*	Tuberculosis

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[a] Chaperone/usher for chaperone–usher and alternate chaperone pathway.

membrane. To accomplish these steps, various adhesive organelles in many different bacteria require two specialized assembly proteins: a periplasmic chaperone and an outer-membrane usher. Chaperone/usher assembly pathways are involved in the biogenesis of over 30 different structures, including composite pili, thin fibrillae, and nonfimbrial adhesins. Here, we will focus on the structure and assembly mechanisms of the prototypical P and type 1 pilus chaperone/usher systems.

Molecular Architecture

P and type 1 are both composite structures consisting of a thin fibrillar tip joined end to end to a right-handed helical rod (**Figures 1(a)–(f)**). Chromosomally located gene clusters that are organizationally and functionally homologous encode P and type 1 (**Figure 1(g)–1(h)**). P pili are encoded by the *pap* (pilus associated with pyelonephritis) gene cluster, whereas type 1 are encoded by the *fim* gene cluster. The P pilus tip is a 2-nm-wide structure composed of a distally located adhesin (PapG), a tip pilin (PapE), and adaptor pilins (PapF and PapK). The PapG adhesin binds to Galα(1-4)Gal moieties present in the globoseries (GbO4) of glycolipids found on the surface of erythrocytes and kidney cells. Consistent with this binding specificity, P pili are major virulence factors associated with pyelonephritis caused by uropathogenic *E. coli*. The minor pilin PapF is considered to join the PapG adhesin to the tip fibrillum, the bulk of which is made up of a polymer of PapE subunits. PapK is believed to terminate the growth of the PapE polymer and to join the tip structure to the rod. The pilus rod is composed of

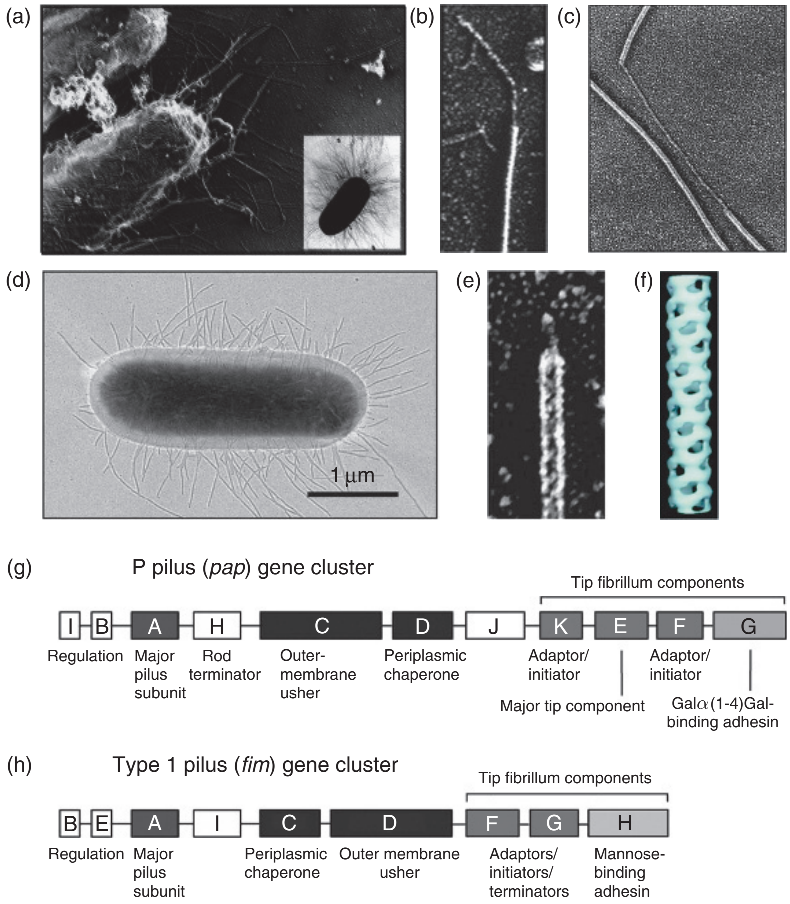
(a)
(b)
(c)
(d)
1 μm
(e)
(f)
(g)
P pilus (*pap*) gene cluster
Tip fibrillum components
I
B
A
H
C
D
J
K
E
F
G
Regulation
Major pilus subunit
Rod terminator
Outer-membrane usher
Periplasmic chaperone
Adaptor/ initiator
Major tip component
Adaptor/ initiator
Galα(1-4)Gal-binding adhesin
(h)
Type 1 pilus (*fim*) gene cluster
Tip fibrillum components
B
E
A
I
C
D
F
G
H
Regulation
Major pilus subunit
Periplasmic chaperone
Outer membrane usher
Adaptors/ initiators/ terminators
Mannose-binding adhesin

Figure 1 P and type 1 architecture and genetic organization. (a) High-resolution electron micrograph of *Escherichia coli* expressing P pili. The inset shows a negatively stained P piliated *E. coli* bacterium as seen by standard transmission electron microscopy. (b) The tip fibrillum structure of a P pilus. (c) P pili are able to unravel into linear fibers, a phenomenon that may help prevent their breakage under high shearing forces or other stresses. (d) *E. coli* expressing type 1. (e) a high-resolution view of the type 1 pilus tip fibrillum, and (f) a three-dimensional reconstruction of a type 1 pilus rod. The images shown are at different magnifications. The P (*pap*) and type 1 (*fim*) pilus gene clusters are depicted in (g) and (h), respectively. These gene clusters share organizational as well as functional homologies. Photos in (d) and (f) were provided by G. Capitani (Capitani G, Eidam O, Glockshuber R, and Grütter MG (2006). *Microbes and Infection* 8: 2284–2290) and S. Müller (Hahn E, Wild P, Hermanns U, Sebbel P, Glockshuber R, Häner M, Taschner N, Burkhard P, Aebi U, and Müller SA (2002). *Journal of Molecular Biology* 323: 845–857), respectively, with permission from Elsiever Ltd.

multiple PapA subunits joined end to end and then coiled into a right-handed, 6.8-nm-thick helical rod, having a pitch distance of 24.9 Å and 3.28 subunits per turn. The rod is terminated by a minor subunit, PapH, which may serve to anchor the pilus in the membrane.

Similar to the P pilus structure, the type 1 pilus has a short, 3-nm-wide fibrillar tip made up of the mannose-binding adhesin, FimH, and two additional pilins, FimG and FimF. The FimH adhesin mediates attachment to mannosylated receptors expressed on a wide variety of cell types and has been shown to be a significant virulence determinant for the development of cystitis. Like other pilus-associated adhesins, FimH has two distinct domains, a C-terminal pilin domain that links the adhesin to the pilus and an N-terminal receptor-binding or lectin domain (**Figure 2**). The adhesin domain of FimH is an elongated 11-stranded β-barrel with an overall jellyroll-like topology and a tip-localized mannose-binding pocket. The type 1 tip fibrillum is joined to a rod comprising predominantly of 500–3000 FimA subunits arranged in a 6- to 7-nm-diameter helix with a pitch distance of 23.1 Å and 3.4 subunits per turn. Both type 1 and P pilus rods have central axial holes with diameters of 2–2.5 and 1.5 Å, respectively. Despite architectural similarities, type 1 appear to be more rigid and prone to breaking than P pili.

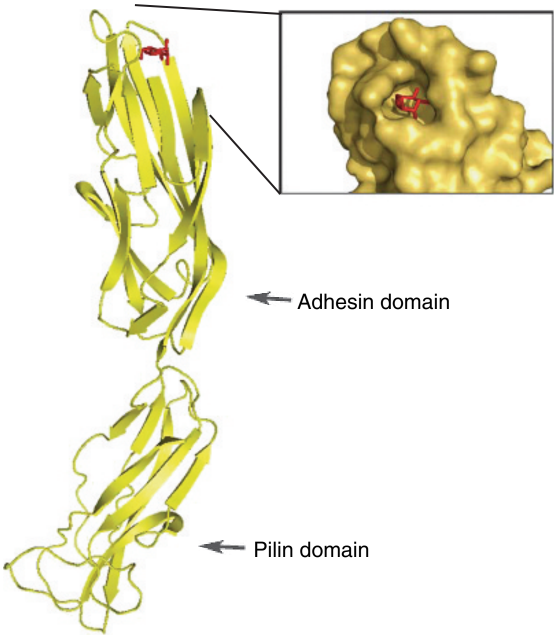

Figure 2 Structure of the type 1 pilus adhesin FimH. Shown on the left is a ribbon model of the FimH adhesin, consisting of two distinct domains. The pilin domain has an immunoglobulin-like fold and allows for incorporation of FimH into the pilus tip. The adhesin domain consists of an 11-stranded β-barrel with an interrupted jellyroll-like motif. This domain is about 50 Å in length and has a distally localized pocket that binds D-mannose (red). A surface model of the mannose-binding pocket of FimH, with bound D-mannose (red), is shown on the right.

In both P and type 1, the major pilin subunits comprising the rods are organized in a head-to-tail manner. Additional quaternary interactions between subunits in adjacent turns of the helical rod appear to stabilize the structure and may help drive the outward growth of the organelle during pilus assembly (see below). Disruption of the latter interactions by mechanical stress or by incubation in 50% glycerol can cause the pilus rod to reversibly unwind into a 2-nm-thick linear fiber similar in appearance to the tip fibrillum (**Figure 1(c)**). It has been proposed that the ability of the pilus rods to unwind allows them to support tension over a broader range of lengths. This may help P and type 1 better withstand stress, such as shearing forces from the bulk flow of fluid through the urinary tract, without breaking. Type 1 are able to helically unwind and thereby resist breakage under forces up to 60 pN, which is equivalent to the weight of about 60 red blood cells.

In addition to composite structures exemplified by P and type 1, chaperone–usher pathways also mediate assembly of thin fibrillae such as K88 and K99 pili and nonfimbrial adhesins. K88 and K99 pili are 2- to 4-nm-thick fibers that mediate adherence to receptors on intestinal cells. They are significant virulence factors expressed by enterotoxigenic *E. coli* (ETEC) strains that cause diarrheal diseases in livestock. These pili were given the 'K' designation after being mistakenly identified as K antigens in *E. coli.* In contrast to P and type 1, the adhesive properties of K88 and K99 pili are associated with the major pilus subunits. The receptor-binding epitopes on the individual major pilus subunits are exposed on the pilus surface and are available for multiple interactions with the host tissue. In general, pili with adhesive major subunits, such as K88 and K99 pili, are thin, flexible fibrillar structures. In comparison, pili with specialized adhesive tip structures, such as P and type 1, are relatively rigid and rod-like.

Assembly Model

The assembly of P pili by the chaperone/usher pathway is among the best understood of any pilus assembly pathway. PapD is the periplasmic chaperone and PapC is the outer-membrane usher for the P pilus system. These proteins are prototypical representatives of the periplasmic chaperone and outer-membrane usher protein families. **Figure 3** presents the current model for pilus assembly by the chaperone/usher pathway, as depicted for P pili.

Periplasmic chaperones

The PapD chaperone, the outer-membrane PapC usher, and all of the P pilus structural subunits have typical signal sequences recognized by the *sec* (general secretion) system. The signal sequences are short, mostly hydrophobic

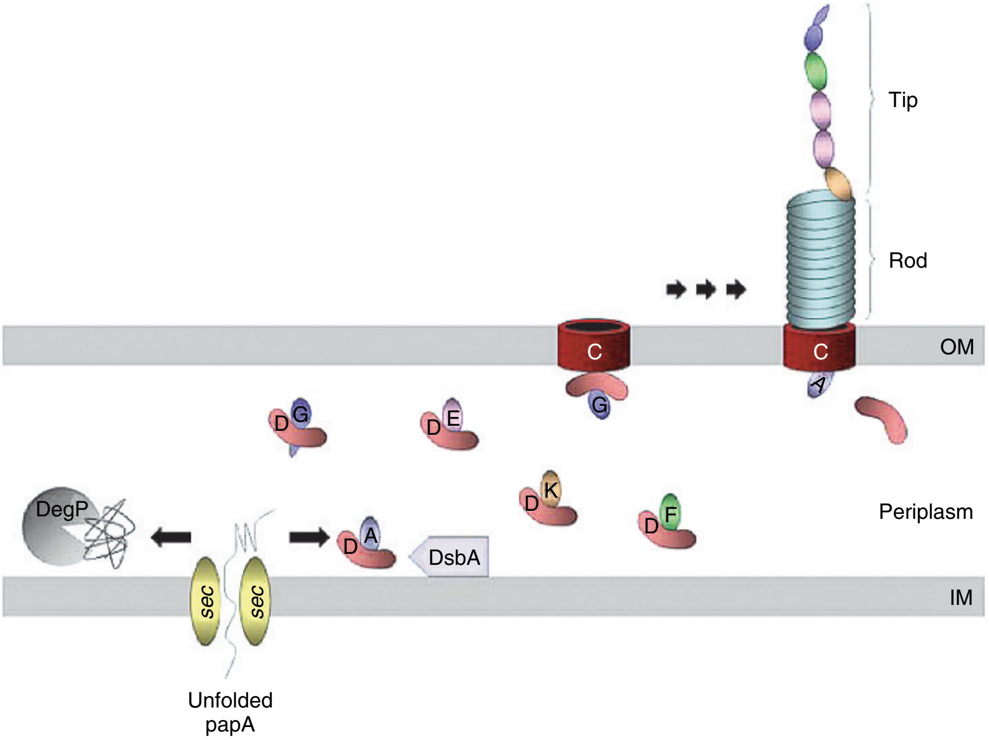

Figure 3 Model of P pilus assembly by the chaperone/usher pathway. Structural subunits for the pilus tip (PapG, PapF, PapE, and PapK) and for the pilus rod (PapA) are translocated across the inner membrane of *Escherichia coli* via the *sec* system. On the periplasmic side of the inner membrane they interact with the chaperone PapD, which facilitates the folding and release of the subunits from the inner membrane. DsbA is required for the proper folding of both the subunits and the PapD chaperone. In the absence of the PapD chaperone, pilus subunits aggregate and are degraded by the periplasmic protease DegP. Subunit–chaperone complexes are targeted to the outer-membrane usher, PapC, where the chaperone is released and subunit–subunit interactions occur. The PapC usher forms a 2-nm-wide pore through which the assembled pilus structure is extruded as a linear fiber across the outer membrane. Once on the exterior of the cell, the linear PapA polymer forms a thick, right-handed helical rod that is unable to slip back through the usher pore. The formation of the coiled PapA rod is thought to help drive the outward growth of the pilus. OM indicates outer membrane and IM indicates inner membrane.

N-terminal motifs that tag proteins for transport across the inner membrane by the *sec* system. This system includes several inner-membrane proteins (SecD-G, SecY, and YajC), a cytoplasmic chaperone (SecB) that binds to presecretory target proteins, a cytoplasmic membrane-associated ATPase (SecA) that provides energy for transport, and a periplasmic signal peptidase. As the P pilus structural subunits emerge from the *sec* translocation machinery into the periplasm, PapD binds to each subunit, facilitating its release from the inner membrane. Each subunit forms an assembly competent, one-to-one complex with PapD. Proper folding of the subunits requires PapD and involves the action of the periplasmic disulfide bond isomerase DsbA. In the absence of PapD, subunits misfold, aggregate, and are subsequently degraded by the periplasmic protease DegP. The misfolding of P pilus subunits is sensed by the Cpx two-component system in which CpxA is an inner-membrane-bound sensor histidine kinase and CpxR is a DNA-binding response regulator. Activation of the Cpx system in response to misfolded pilin subunits or other types of periplasmic stress inhibits P pilus biosynthesis by downregulating pilin expression.

The three-dimensional crystal structures of both the PapD and FimC chaperones have been solved. Both chaperones consist of two Ig (immunoglobulin)-like domains, each consisting of seven β-sheets, oriented into a boomerang shape such that a subunit-binding cleft is created between the two domains. A conserved internal salt bridge is thought to maintain the two domains of the chaperone in the appropriate orientation. Using genetics, biochemistry, and crystallography, PapD was found to interact with pilus subunits in part by binding to a highly conserved motif present at the C-terminus of all subunits assembled by PapD-like chaperones. The finer details of how PapD-like chaperones interact with pilus subunits were unveiled by determining the crystal structures of the PapD–PapK and the FimC–FimH chaperone–subunit complexes (**Figure 4**). This work demonstrated that the PapD and FimC chaperones have similar interactions with their respective subunits. Only the PapD–PapK structure is considered here. PapK has a single domain

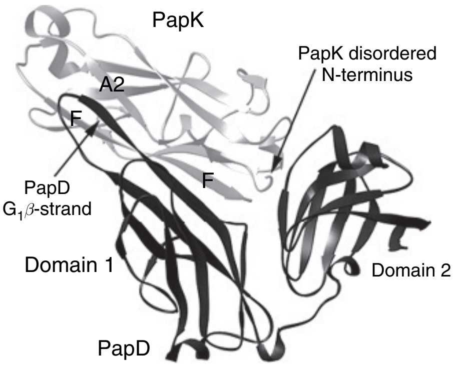

Figure 4 Ribbon model based on the crystal structure of the PapD periplasmic chaperone (in black) complexed with the PapK pilus subunit (in gray). The G_1 β-strand in domain 1 of PapD completes the Ig fold of the pilin, occupying the groove formed between the A2 and F strands of PapK. This interaction has been termed donor strand complementation. The eight N-terminal amino acids of PapK are disordered in the structure. These residues have been implicated in mediating subunit–subunit interactions within the mature pilus organelle. During pilus biogenesis, the N-terminal strand of a pilin can displace the G_1 β-strand of the chaperone and insert into the C-terminal groove of the neighboring subunit. The mature pilus thus consists of a linear array of canonical Ig domains, each of whose fold is completed by a strand from the neighboring subunit.

consisting of an Ig fold that lacks the seventh (C-terminal) β-strand that is present in canonical Ig folds. The absence of this strand produces a deep groove along the surface of the pilin subunit and exposes its hydrophobic core. By an interaction known as donor strand complementation, a G_1 β-strand of the PapD chaperone occupies the groove in PapK and completes the Ig fold. This interaction shields the hydrophobic core of PapK and stabilizes immature pilus subunits within the periplasm. Interactions with PapD-like chaperones also accelerate the folding of pilin subunits about 100-fold, allowing for more rapid assembly of pili. The residues that make up the C-terminal groove formed by subunits and bound by PapD-like chaperones have been shown by mutagenesis and, more recently, by crystallographic studies to be involved in subunit–subunit interactions within the final pilus structure. Thus, in addition to stabilizing immature pilus subunits, the donor strand complementation interaction also caps one of the interactive surfaces of the subunit and prevents premature oligomerization and aggregation of pilus subunits within the periplasm.

Outer-membrane ushers

Once formed in the periplasm, chaperone–subunit complexes are targeted to the outer-membrane usher where the chaperone is released, exposing interactive surfaces on the subunits that facilitate their assembly into the pilus. Studies in the P and type 1 pilus systems have demonstrated that the adhesin–chaperone complexes, PapDG or FimCH, bind tightest and fastest to the usher and that the adhesins are the first subunits assembled into the pilus. Binding of the chaperone–adhesin complex induces a conformational change in the usher, possibly priming it for pilus assembly. Additional subunits are incorporated into the pilus depending, in part, upon the kinetics with which they are partitioned to the usher in complex with the chaperone. Conserved N-terminal regions, in addition to the conserved C-terminal motif of the pilus subunits, mediate subunit–subunit interactions within the mature pilus. Differences in the complementary surfaces in these conserved regions from one subunit to another may help dictate which of the subunits can be joined to one another during pilus assembly. Thus, the order of the subunits within the final pilus structure is determined by the specific contacts made between the different pilus subunits and also by the differential affinities of the various chaperone–subunit complexes for the usher.

In addition to acting as an assembly platform for the growing pilus, the usher protein appears to have additional roles in pilus biogenesis. High-resolution electron microscopy revealed that the PapC usher is assembled into an oligomeric 15-nm-diameter ring-shaped complex with a 2-nm-wide central pore. Each PapC monomer is composed predominantly of transmembrane β-sheets, typical of outer-membrane pore-forming proteins. The N-terminal domain of PapC binds chaperone-complexed subunits, whereas the C-terminal domain stabilizes the chaperone–subunit complexes and is involved in subsequent stages during pilus assembly. Oligomerization of the usher and subsequent pilus biogenesis are both initiated by interactions between the adhesin and the usher. During assembly of type 1, the FimD usher directs the receptor-binding domain of the FimH adhesin into the pore of the usher, whereas the pilin domain remains bound to the chaperone until displaced by the next incoming chaperone–subunit complex. After dissociating from the chaperone at the usher, subunits are incorporated into a growing pilus structure that is predicted to be extruded as a 1-subunit-thick linear fiber through the central pore of the usher complex. Packaging of the linear pilus fiber into a thicker, helical rod on the outside surface of the bacterium may provide a driving force for the translocation of the pilus across the outer membrane, possibly acting as a sort of ratcheting mechanism to force the pilus to grow outward. Combined with the targeting affinities of the chaperone–subunit complexes for the usher and the binding specificities of the subunits for each other, this may provide all the energy and specificity needed for the ordered assembly and translocation of pili across the outer membrane.

Alternate Chaperone Pathway

A variation of the chaperone/usher pilus assembly pathway has been identified in strains of ETEC and a few other pathogenic bacteria. ETEC are major pathogens associated with diarrheal diseases of travelers, infants, and young children. Strains of ETEC produce several types of uniquely assembled adhesive pili that are considered to be important mediators of bacterial colonization of the intestine. The best studied of these pili is coli surface antigen 1 (CS1), which appears to be composed predominantly of a major subunit, CooA, with a distally located minor component, CooD, that is required for ETEC adherence to host cells. Several CS1-like pili have been identified and include CS2, CS4, CS14, CS17, CS19, CFA/I (colonization factor I, expressed by various ETEC strains), Tcf (expressed by *Salmonella enterica* serovar Typhi), and the cable type II pili of *Burkholderia cepacia*, an opportunistic pathogen of cystic fibrosis patients. Four linked genes, *CooA, CooB, CooC*, and *CooD*, are the only specific genes required for the synthesis of functional CS1. Electron microscopic examination reveals that the CS1-like pili are architecturally similar to P and type 1 assembled by the chaperone/usher pathway (**Figures 5(a)** and **5(b)**), although none of the proteins involved in the biogenesis of CS1-like pili have any significant sequence homologies to those of any other pilus system.

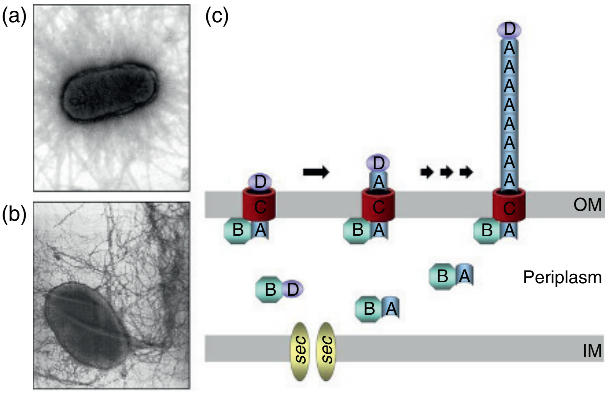

Figure 5 Pili assembled by the alternate chaperone pathway. Transmission electron micrographs show (a) CS2 pili (~2 nm thick) assembled by the alternate chaperone pathway in *Escherichia coli* (photo courtesy of Harry Sakellaris and June R. Scott) and (b) Cbl pili of *Burkholderia cepacia*. Reproduced from Sajjan US, Sun L, Goldstein R, and Forstner JF (1995). *Journal of Bacteriology* 177: 1030–1038, with permission from ASM press. (c) Assembly of CS1 from *E. coli* via the alternate chaperone pathway. The CooB chaperone forms periplasmic complexes with the main components of the pilus, CooA and CooD. CooB also appears to bind and stabilize the outer-membrane protein CooC during pilus assembly. CooC functions as an outer-membrane (OM) channel for passage of the pilin fiber.

The assembly of CS1-like pili is functionally similar to that of P pili but depends on a specialized set of periplasmic chaperones that are distinct from those of the chaperone/usher pathway described above. Therefore, this mode of pilus assembly is referred to as the alternate chaperone pathway. In the case of CS1 the chaperone CooB binds to and stabilizes the major and minor pilin subunits, CooA and CooD, which enter into the periplasm in a *sec*-dependent fashion (**Figure 5(c)**). Both CooA and CooD share a conserved sequence motif near their C-termini that may function as a chaperone recognition motif. One of the functions of CooB appears to be the delivery of the pilin subunits to an outer-membrane protein, CooC, which may function as a channel, or usher, for the assembly of pilus fibers. In addition to the pilin subunits, CooB also binds to and stabilizes CooC in the outer membrane. In the absence of the CooB chaperone, CooC and the pilin subunits are degraded. The minor subunit CooD localizes to the tip of the CS1 pilus and is required for secretion of the major pilus subunit CooA. Expression of CooD also seems to positively regulate the number of pili produced, in keeping with a model in which the positioning of a minor tip subunit (CooD in this case) by an outer-membrane usher complex is required to initiate assembly of the pilus shaft. As CS1-like pili do not appear to be related to those assembled by the chaperone/usher pathway, it has been suggested that these two pilus assembly systems arose independently through convergent evolution.

Type II Secretion Pathway for Type IV Pilus Assembly

Type IV pili are multifunctional, strong, and flexible filamentous structures expressed by a wide diversity of bacteria, including a number of animal and plant pathogens. These include *Pseudomonas aeruginosa, Pseudomonas stutzeri, Neisseria gonorrhoeae, Neisseria meningitidis, Eikenella corrodens, Legionella pneumophila, Ralstonia solanacearum, Myxococcus xanthus, Francisella novicida, Francisella tularensis, Dichelobacter nodosus, Synechocystis* spp., *Moraxella* spp., and *Azoarcus* spp. Type IV pili can promote bacterial survival and pathogenesis by mediating bacterial interactions with animal, plant, and fungal cells, in some cases contributing to bacterial invasion of target host cells. In addition, these pili can modulate target cell specificity, function in DNA uptake, promote bacterial autoaggregation and biofilm formation, and act as receptors for bacteriophage. Type IV pili are also associated with a flagella-independent form of bacterial locomotion called twitching motility (also known as social motility in *Myxococcus* spp.) that allows for the lateral spread of bacteria across a surface.

Type IV pili are 6 nm in diameter and can extend up to several micrometers in length. These pili are typically assembled at one pole of the bacterium and can withstand forces up to 100 pN. Based on the length and amino acid sequence of the pilin subunits, type IV pili can be divided into two subclasses: type IVa and type IVb. Type IVa pilin subunits are usually composed of 145–160 amino acids. These subunits have several distinctive features, including a short (5–6 amino acids), positively charged leader sequence that is cleaved during assembly, *N*-methyl-phenylalanine as the first residue of the mature subunit, and a highly conserved, hydrophobic N-terminal domain that forms the core of the mature pilus.

Type IVb pili include the toxin coregulated pilus (TcpA) of *Vibrio cholera*, bundle-forming pilus (BfpA) of enteropathogenic *E. coli* (EPEC), longus (LngA) and CFA/III pili of ETEC, conjugative R64 thin pilus of *E. coli*, and colonization factor Citrobacter (CfcA) of *Citrobacter rodentium*. In contrast to type IVa pilins described above, the known type IVb pilins are somewhat larger (180–238 amino acids) and have a longer (15–30 amino acids) leader sequence. Also, in place of *N*-methyl-phenylalanine as the first amino acid in the mature pilus subunit, type IVb subunits have other methylated residues such as *N*-methyl-methionine for TcpA and *N*-methyl-leucine for BfpA. TCP, BFP, and longus pili form large polar bundles over 15 μm in length. In contrast, CFA/III pili are 1–10 μm long and are peritrichously expressed.

Atomic resolution structures of the type IVa pilins PilE (*N. gonorrhoeae*) and PilA (*P. aeruginosa* strains K and K122-4), as well as the type IVb pilin TcpA (*V. cholera*), have been solved (**Figures 6** and **7**). Based on these structures, all type IV pilin subunits are predicted to have a fairly similar structure consisting of a conserved N-terminal α-helical hydrophobic core surrounded by β-sheets and a hypervariable C-terminus. The core α-helix of type IV pilins, known as $\alpha 1$ (blue in **Figure 6**), is usually composed of 53 amino acids and is divided into a protruding hydrophobic N-terminal half ($\alpha 1$-N, residues 1–29) and an amphipathic C-terminal half ($\alpha 1$-C, residues 30–53). The hydrophobic face of $\alpha 1$-C is wrapped by antiparallel β-sheets to form a globular head domain, whereas the hydrophilic face is buried within the assembled pilus. This hydrophobic packing of the inner core of α-helices along with the flexibility of these helices may permit type IV pili to bend and adopt twisted, bundled conformations (**Figure 7**). Hydrogen bonds throughout the layer of β-sheets may provide much of the mechanical stability for the pilus.

Type IV pilin globular head domain (green in **Figure 6**) is flanked by two highly variable regions designated the $\alpha\beta$-loop (orange in **Figure 6**) and D-region (yellow in **Figure 6**). The $\alpha\beta$-loop functions as a linker between the $\alpha 1$ and the β-sheets, and facilitates pilin–pilin interactions before and after assembly. The D-region contains a conserved disulfide bridge, which is essential for pilus assembly. These hypervariable regions associate with the layer of β-sheets through only a few conserved interactions. Thus, they can be structurally pliant and accommodate extreme amino acid changes that lead to antigenic variation and altered binding specificities without disrupting the assembly of the pilus. The antigenic characteristics of type IV pili synthesized by *N. gonorrhoeae* can be modified extensively by a remarkable mechanism. This pathogen encodes more than 15 different silent pilin genes termed PilS that lack the invariant N-terminal domain present in PilE. By recombination of silent PilS genes with the PilE locus, a single neisserial strain can theoretically express more than 10 million PilE variants. This antigenic variation helps *N. gonorrhoeae* evade the host immune system, allowing the establishment and maintenance of infection.

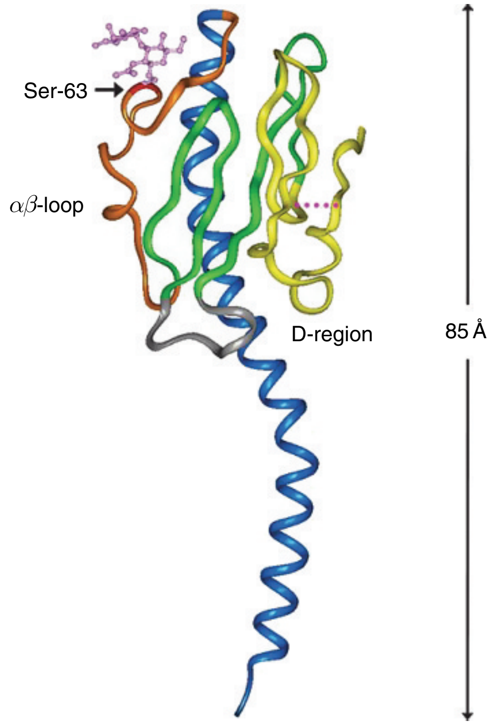

Figure 6 Ribbon model of the type IVa pilin, PilE, from *Neisseria gonorrhoeae*. Secondary structural elements include a hydrophobic core surrounded by β-sheets (green), a conserved N-terminal α-helical spine (blue) connected to a variable domain containing an $\alpha\beta$-loop (orange) with an O-linked disaccharide at Ser-63 and a disulfide-containing C-terminal D-region (yellow). The conserved disulfide bridge in the D-region is signified by a dotted line (magenta).

The biogenesis of type IV pili is substantially more complicated than pilus assembly by the chaperone/usher or alternate chaperone pathways. Most type IVa pili-encoding genes are located on the chromosome, whereas most type IVb pili are encoded by plasmids. The number of genes essential for type IV pilus biogenesis and function ranges from 14, for pili such as BFP, to over 20, for structures such as the type IVa pili of *N. gonorrhoeae*. In *P. aeruginosa*, it is estimated that about 0.5% of the

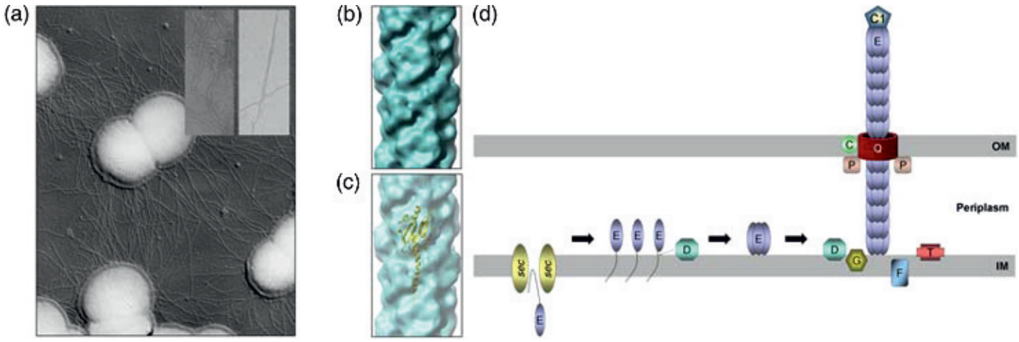

Figure 7 Type IV pili. (a) Scanning electron micrograph of *Neisseria gonorrhoeae* diplococci expressing peritrichous type IV pili. Insets show close-up views of the pili. (b) Three-dimensional reconstruction of type IV pilus rod, which is made up of repeating and overlapping PilE pilin subunits. (c) Localization of a single PilE subunit (yellow) within the pilus rod. Image courtesy of L. Craig and C. Brinton (Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, and Tainer JA (2006). *Molecular Cell* 23: 651–662), with permission from Elsiever Ltd. (d) Model of type IV pilus assembly by *N. gonorrhoeae*. The PilE prepilin is translocated into the periplasm aided by the *sec* machinery. PilE is processed by the PilD signal peptidase, which cleaves the positively charged leader sequence from the N-terminus of the pilin subunit. An inner-membrane assembly complex then assembles the mature PilE subunit into a pilus fiber. PilQ mediates translocation of the pilus through the outer membrane, possibly with the assistance of other factors such as PilP. PilC associates with the pilus and may act as a restraining clip, preventing PilT-mediated retraction of the type IV pilus rod. The PilC1 adhesin, which appears to be incorporated at the tip of the growing pilus fiber, also seems to be required for translocation of the pilus through the outer membrane.

bacterium's genome is involved in the synthesis and function of type IV pili. Among the various bacterial species expressing type IV pili, the genes encoding the type IV pilus structural components tend to be similar, whereas the regulatory components surrounding them are typically less conserved.

Type IV pilus biogenesis occurs in three steps: fiber formation, fiber stabilization, and surface localization of the intact organelle. In the case of *N. gonorrhoeae* pili, the process requires more than 14 assembly proteins and a type II secretion system-related multicomponent assembly complex. Components include a prepilin peptidase (PilD) that cleaves off the leader peptide from nascent pilin subunits; a polytopic inner-membrane protein that may act as a platform for pilus assembly; a hydrophilic nucleotide-binding protein located in the cytoplasm or associated with the cytoplasmic face of the inner membrane that may provide energy for pilus assembly; and an outer-membrane protein complex (PilQ) that forms a pore for passage of the pilus to the exterior of the bacterium.

The PilE/PilA propilin subunits are transported into the periplasm by the *sec* translocation machinery (**Figure 7(d)**). After translocation, the propilin subunits remain anchored in the inner membrane by their hydrophobic N-terminal α-helical domains, with their hydrophilic C-terminal heads oriented toward the periplasm. Removal of the positively charged propilin leader sequence by the PilD signal peptidase drives the hydrophobic stems of the pilin subunits to associate and form a pilus. An inner-membrane assembly complex made up of several proteins including PilD, PilF, PilG, and PilT aids in this process. The outer-membrane-associated PilC has been shown to stabilize the nascent pilus before translocation and prevent PilT-mediated retraction. The assembled pilus penetrates the outer membrane through an electrochemically gated oligomeric channel formed by PilQ. The ring-shaped oligomeric channel formed by PilQ has 12-fold symmetry and an internal diameter of 5–7 nm. Its assembly and localization is facilitated and stabilized by small lipoproteins, PilP in *N. gonorrhoea*, BfpG in EPEC, and Tgl in *M. xanthus*. The PilC1 adhesin associated with the tips of type IV pili in *N. gonorrhoea* may facilitate passage of the nascent pili through the PilQ pore.

One implication of this assembly model is that the N-terminal region of PilA/PilE resides in a continuous hydrophobic environment during both inner-membrane transport and pilus assembly. This may allow polymerization and, interestingly, depolymerization of the pilus to proceed with only minimal energy requirements. Type IV pili undergo rounds of extension and retraction by polymerization and depolymerization reactions. The nucleotide-binding protein PilB facilitates the polymerization and extension of pili, whereas PilT (a distant member of the AAA ATPase motor family) mediates rapid depolymerization and retraction, at rates up to 1500 pilin subunits per second. These rounds of extension and retraction are the basis of twitching motility, one of the functions of type IV pili. The current model for twitching motility can be compared to the usage of a grappling hook. It involves the extension of a pilus, the adhesion of the pilus tip to a surface, and then the retraction of the pilus to pull the cell forward. The capacity of type IV pili to retract may also provide a means for transforming DNA, which could potentially interact with the type IV pilus, to enter into the bacterial cell.

Mutation in components of the type IV pilus PilT in *N. gonorrhoea* results in the loss of transformability. Naturally competent bacteria such as *Bacillus subtilis, H. influenzae*, and *S. pneumoniae* do not have type IV pili, but do have similar components that facilitate the transformation of naked DNA. The adhesive properties of type IV pili are, in general, determined by the major pilus subunit. Additional minor components, however, may associate with these pili and alter their binding specificities. In the case of *Neisseria*, a tip-localized adhesin, PilC1, appears to mediate bacterial adherence to epithelial cells.

Many of the components involved in type IV pilus assembly share homology with proteins that are part of DNA uptake and protein secretion systems, collectively known as the main terminal branch of the general secretory (*sec*-dependent) pathway, or type II secretion. Type II secretion systems transport a variety of proteins, including toxins, proteases, cellulases, and lipases, from the periplasm to the extracellular space. The pullulanase secretion system in *Klebsiella oxytoca* is a prototypical type II secretion system. Transport of pullulanase (PulA, a starch-hydrolyzing lipoprotein) through the outer-membrane protein complex (PulD) involves four type IV pilin-like proteins, known as pseudopilins. Like type IV pilins, the pseudopilins are processed by a prepilin peptidase; the peptidase is even interchangeable between the two systems. The pseudopilins may assemble into a 'secretion tube' – a pilus-like structure that extends across the periplasm and facilitates PulA transfer to the outer membrane. Pseudopilin filaments have been observed upon overexpression of the pseudopilin genes, but have not yet been demonstrated to exist under normal conditions.

Another structure that is believed to have evolutionary relatedness to type IV pili is the archaeal flagellum. Archaeal flagella are about 10 nm in diameter, and, like bacterial flagella, rotate and switch directions to mediate motility. However, orthologues of bacterial flagellin motor proteins have not been found in any archaeal genome, and the mechanism of rotation and switching in archaea is still unknown. Archaeal flagellar subunits have no similarity to bacterial flagellins, but they are similar to type IV pilins. Like type IV pilins, archaeal flagellins require processing by a signal peptidase before assembly into a filament, and their assembly requires both a nucleotide-binding protein and a polytopic cytoplasmic membrane protein.

Conjugative Pilus Assembly Pathway

In Gram-negative bacteria, certain pili, collectively known as conjugative pili, facilitate the transfer of DNA among bacteria. These pili allow donor and recipient bacteria to make specific and stable intercellular contacts before DNA transfer is initiated. DNA is moved between cells through the mating pair formation system, which is related to the so-called type IV secretion systems (T4SS). Horizontal gene transfer, or conjugation, mediated by conjugative pili is inextricably associated with the spread of antibiotic resistance among bacterial pathogens. Conjugative pili are generally encoded by self-transmissible plasmids that are capable of passing a copy of their genes to a recipient bacterium. Closely related plasmids, with similar replication control systems, are unable to coexist within the same cell. This property has been termed 'incompatibility' and provides the primary basis for cataloguing conjugal plasmids and the pili that they encode. Thus far, in *E. coli* alone, over 25 incompatibility groups composed of well over 100 different plasmids have been defined. Plasmids within a particular incompatibility group usually encode conjugative pili with similar antigenic properties, sensitivities to pilus-specific phage, and morphologies.

Among the multitude of known incompatibility groups, three morphologically and functionally distinct types of conjugative pili have been defined: (1) rigid; (2) thick, flexible; and (3) thin, flexible pili. Rigid conjugative pili are 8- to 11-nm-wide structures that are usually specified by conjugal DNA transfer systems that function well only on solid surfaces. Thick, flexible pili, on the other hand, are 8- to 11-nm-wide structures that typically, but not always, promote conjugation on solid surfaces and in liquid media equally well. Conjugal DNA transfer promoted by rigid or thick, flexible pili can be enhanced, in some cases, by the presence of thin, flexible pili. These pili are similar in appearance to type IV pili and at least one member of the thin, flexible pilus group (the R64 thin pilus) has been identified at the molecular level as a type IVb pilus (as described above). Thin, flexible pili appear to function primarily in the stabilization of bacterial mating pairs, increasing the rate of DNA transfer. Conjugation does not occur in the presence of thin, flexible pili alone or in the absence of rigid or thick, flexible pili.

The most thoroughly studied conjugative pilus is the F pilus encoded by the self-transmissible, broad host range F (fertility) plasmid, a member of the F1 incompatibility (IncF1) group of plasmids borne by *E. coli*. The F pilus system is prototypical for numerous other conjugation systems, and F pilus biogenesis is distinct from type IV and other pilus assembly pathways. F pili are 8-nm-thick, flexible helical filaments that consists primarily, if not completely, of repeating 7.2 kDa (70 amino acid) TraA pilin subunits. Donor (F^+) cells typically express 1–3 F pili that are usually 1–2 μm long. Each F pilus possesses a 2-nm-wide central channel that is lined by basic, hydrophilic residues, which could potentially interact with negatively charged DNA or RNA molecules during conjugation. TraA is organized into pentameric, doughnut-like disks that are stacked within the pilus such that successive disks are translated 1.28 nm along the pilus axis and rotated 28.8° with respect to the lower disk. The TraA pilin has two hydrophobic domains located toward the center and at the C-terminus of the pilin. The hydrophobic domains are thought to extend as antiparallel α-helices from the central axis to the periphery of the pilus shaft. These domains are separated by a short, basic region that appears to form the hydrophilic wall of the central channel of the pilus. The N-terminal domain of TraA is predicted to face the exterior of the pilus. However, this domain is antigenically masked when the N-terminal residue of TraA is acetylated during maturation of the pilin (see below). This modification is common among all known F-like pilins and appears to cause the N-terminal domain to be tucked back into or along the pilus shaft. Acetylation is not essential for F pilus assembly or function, but does help prevent aggregation of F-like pili and affects the phage-binding characteristics of these organelles. Phage are also known to recognize the C-terminal hydrophobic domain of TraA. Although masked within the pilus shaft, the acetylated N-terminal domain of TraA appears to be exposed in unassembled pilin subunits and at the distal tips of pili. The F pilus tip is believed to initiate contact between donor and recipient cells during conjugation and serves as a receptor for F-specific filamentous phage. Alterations in the N-terminal sequence of pilin subunits provide the primary basis for the antigenic diversity observed among F-like pili expressed by plasmids F, R1-19, ColB2, pED208, and R100-1.

A number of genes encoded by the F plasmid *tra* operon are required for conjugation. TraA is synthesized as a 12.8 kDa (121 amino acids) cytoplasmic propilin, but is processed into a 7.2 kDa pilin form by host signal peptidase LepB in the periplasm. Pilin maturation gene products TraQ and TraX mediate the processing of the TraA pilin to its mature form. TraQ, a chaperone-like inner-membrane protein, escorts TraA into the inner membrane and helps position the TraA propilin for processing into mature pilin. In the absence of TraQ, the translocation of TraA is disrupted and most of the pilin subunits are degraded. After processing, the N-terminal residue (alanine) of TraA is acetylated by TraX, a polytopic inner-membrane protein. The core T4SS proteins, TraB, TraC, TraE, TraG, TraK, TraL, and TraV, are involved in both pilus assembly and DNA transport. Additional gene products (TraW, TraN TraU, TraF, TraH, TrbC, and TrbI) affect the assembly of TraA into the pilus filament. Most of these proteins appear to associate with either the inner or outer bacterial membrane and may constitute a pilus assembly complex that spans the periplasmic space.

The exact mechanism by which TraA is assembled into pili is not yet defined. Pools of up to ~100 000 mature TraA pilins accumulate in the inner membrane before pilus assembly, which appears to occur by addition of pilin subunits at the base of the growing pilus. Both hydrophobic domains of TraA span the inner membrane, with the hydrophilic region of TraA connecting them on the cytoplasmic side. Small clusters of TraA also accumulate in the outer membrane and these may function as intermediates in F pilus assembly and disassembly. Large regions of the TraA sequence have the propensity to assume both β-sheet and α-helical structures, although the α-helical conformation is known to predominate in assembled pili. It has been suggested that a shift between β-sheet and α-helical conformations drives pilus assembly and disassembly. F pilus assembly is energy dependent and the depletion of ATP levels by respiratory poisons such as cyanide results in F pilus depolymerization and retraction. It is possible that TraA is normally cycled between pili and periplasmic/inner-membrane pools by rounds of pilus outgrowth and subsequent retraction. During conjugation, F pilus retraction is believed to serve a stabilizing function by shortening the distance between bacterial mating pairs and allowing for more intimate contact.

Several components of the F pilus assembly machinery share significant homology with proteins encoded by other conjugative systems. These include proteins specified by broad host range plasmids in other incompatibility groups (such as IncN, IncP, and IncW) and many of the proteins encoded by the Ti (tumor inducing) or Ri (root inducing) plasmid-specific *vir* genes of the plant pathogens, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively. These bacteria elaborate 10-nm-wide promiscuous conjugative pili, called T pili, at one end of a cell that direct the interkingdom transfer of a specific genetic element, known as T-DNA, into plant and yeast cells. The introduction of T-DNA into plant cells induces plant tumor formation. The exact mechanism of T-DNA transfer is not known, but the T pilus is essential for T-DNA transfer. T pili may facilitate direct cell-to-cell contact for T-DNA transfer, possibly providing a conduit for passage of T-DNA. In addition, the T pilus could act as a sensor, allowing infecting bacteria to receive signals from the host plant. T pilus assembly by *A. tumefaciens* requires the expression of at least 12 *vir* gene products encoded by the Ti plasmid. VirB2 is the major, and possibly only, component of the T pilus and it is predicted to be structurally homologous to the F pilus subunit TraA. Unlike F pili, T pili do not retract, but instead wind together to form very compact coils, bringing the surface of bacterium and the host closer (**Figure 8**).

Other than possibly stabilizing donor–recipient interactions, it is not yet clear as to how F and T pili or any pilus structures function in conjugative DNA transfer processes. However, at least in F pili, substantial evidence suggests that pilus components or the pilus itself can serve as a specialized channel for the transmission of DNA and any accompanying pilot proteins across the donor and possibly the recipient cell membranes. In light of this

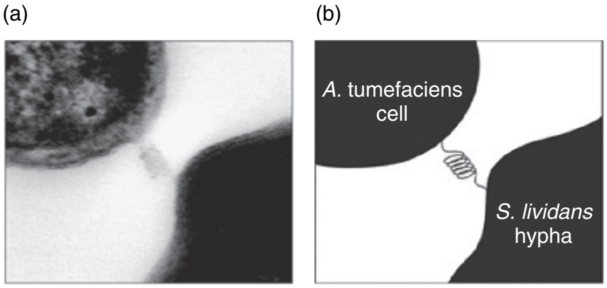

Figure 8 T pilus-mediated attachment of *Agrobacterium tumefaciens* to *Streptomyces lividans*. For clarity, a schematic representation of the electron micrograph showing a coiled T pilus linking the two microbes in (a) is shown in (b). Reproduced from Lai EM and Kado CI (2000). *Trends in Microbiology* 8: 361–369, with permission from Elsevier Ltd.

possibility, it is interesting to note that many components of the conjugative pilus systems encoded by the IncF, IncN, IncP, and IncW plasmids and by the *vir* genes of *A. tumefaciens* are similar to the Ptl proteins responsible for the export of the multiple subunit toxin of *Bordetella pertussis*. Interestingly, in *Brucella suis* and *Bartonella henselae*, virB homologues are arranged in the same order as in the *A. tumefaciens virB* operon. Furthermore, these secretion systems seem to be distantly related to transport systems used by *L. pneumophila, Helicobacter pylori*, and *Rickettsia prowazekii* to inject virulence factors into host eukaryotic cells. Conjugative pilus systems such as those encoding F and T pili thus appear to be representative of a larger family of macromolecular transport systems. These so-called type IV secretion systems represent a major pathway for the transfer of both nucleic acid and proteins between cells. Understanding how conjugative pili help mediate the intercellular transfer of macromolecules remains a significant challenge.

Extracellular Nucleation/Precipitation Pathway

Many strains of enteric bacteria, including *E. coli* and *Salmonella* species, produce a class of thin (<2 nm), irregular, and highly aggregated surface fibers known as curli (**Figures 9**(**a**) and **9**(**b**)). Curli are described as amyloid-like fibers, and are composed mainly of extracellular matrix components, namely protein, cellulose, and an unknown polysaccharide. They are highly stable structures and extreme chemical treatments are required to depolymerize them. The major component of *E. coli* curli is a 15.3 kDa protein known as CsgA, which shares over 86% primary sequence similarity to its counterpart in *S. enterica* ser. Typhimurium, AgfA.

The formation of curli represents a departure from the other modes of pilus assembly discussed in the

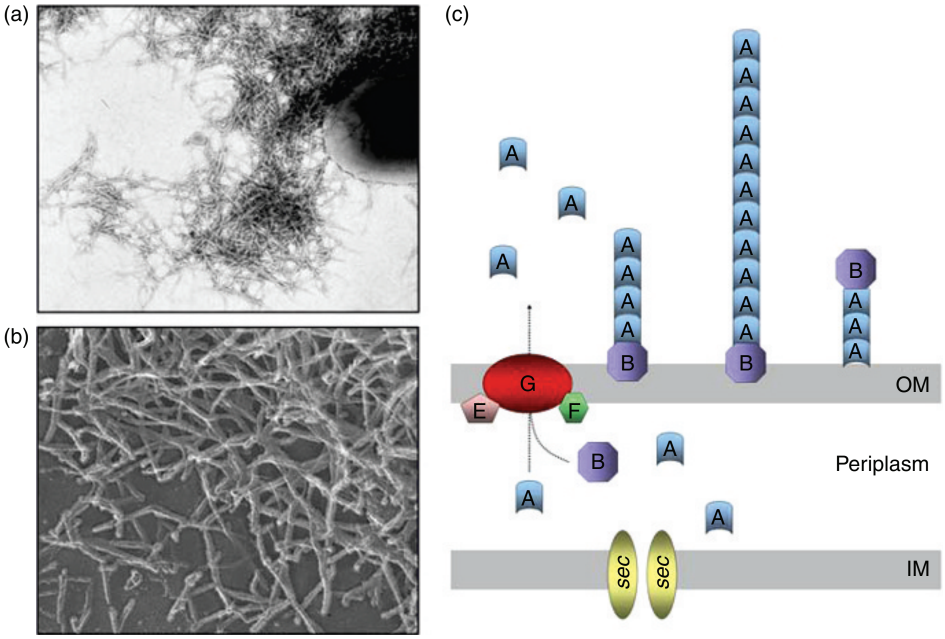

Figure 9 Curli assembly by an extracellular nucleation/precipitation pathway. (a) Electron micrographs of negatively stained *Escherichia coli* expressing curli. (b) High-resolution electron micrograph, obtained using a deep-etch technique, of purified curli. Photo courtesy of M. Chapman (Barnhart MM and Chapman MR (2006). *Annual Review of Microbiology* 60: 131–147), with permission from Annual Reviews. (c) Model of curli assembly by the extracellular nucleation/precipitation pathway. CsgA, the main component of curli from *E. coli*, is secreted across the outer membrane. CsgB serves to nucleate CsgA assembly. CsgG is an outer-membrane-localized lipoprotein that is required for the secretion of CsgA and CsgB, although its function is not yet entirely clear. CsgE and CsgF are periplasmic proteins that interact with CsgG at the outer membrane.

previous sections. Whereas structures exemplified by P, CS1, type IV, and F pili are assembled from the base, curli formation occurs on the outer surface of the bacterium by the precipitation of secreted soluble pilin subunits into thin fibers (**Figure 9**). In *E. coli*, the products of two divergently transcribed operons are required for curli assembly. The *csgBA* operon encodes the primary fiber-forming subunit, CsgA, which is secreted as an unpolymerized protein directly into the extracellular environment. The second protein encoded by the *csgBA* operon, CsgB, is proposed to induce polymerization of CsgA at the cell surface. In support of this model, by interbacterial complementation it has been demonstrated that a $CsgA^+CsgB$-donor strain can secrete CsgA subunits that can be assembled into curli on the surface of a $CsgA^-CsgB^+$-recipient strain. In the absence of CsgA, overexpressed CsgB is able to form short polymers on the bacterial cell surface.

The *csgDEFG* operon encodes a gene for a transcriptional activator of curli synthesis (CsgD), and three genes encoding putative assembly factors. One of these factors, CsgG, has recently been shown to be a lipoprotein that is localized to the outer membrane. In the absence of CsgG, curli assembly does not take place, and CsgA and CsgB are rapidly degraded. The precise role of CsgG is not known at this time. It has been suggested that CsgG might act as a chaperone that facilitates the secretion of the CsgA and CsgB and protects them from degradation within the periplasm. It is also possible that CsgG assembles into oligomeric, ring-shaped complexes that could function as a Csg-specific channel within the outer membrane. It has been reported that a strain lacking CsgE is defective in curli assembly, as the stability of both CsgA and CsgB in this mutant background is greatly reduced. Mutation of *csgF* does not affect CsgA secretion, but CsgA made by *csgF* mutants does not polymerize. Environmental factors such as very low salt concentrations, nutrient limitation, microaerophilic conditions, and temperatures below 30 °C favor maximal curli gene expression. RpoS, a stationary-phase sigma factor; OmpR/EnvZ, a two-component regulatory system; and IHF, a global regulatory protein all positively regulate curli expression. Two other two-component regulatory systems, CpxA/R and Rcs, negatively regulate curli expression. Interestingly, histone-like protein HN-S positively regulates curli expression in *S. enterica* ser. Typhimurium, but negatively regulates it in *E. coli.*

Curli promote bacterial adherence to host cells and tissues by binding to a variety of host proteins, including plasminogen, fibronectin, and human contact phase proteins. Interestingly, curli are recognized as a pathogen-associated molecular pattern by host Toll-like receptor 2, a component of the innate immune system that can recognize incoming pathogens and induce robust proinflammatory responses. Curli are also involved in bacterial colonization of inert surfaces, and have been implicated in cell aggregation and biofilm formation.

Type III Secretion Pathway

The various pilus assembly pathways described in the previous sections all rely on components of the *sec* machinery for the translocation of their respective pilus subunits across the inner membrane. Other types of pili are assembled by a *sec*-independent pathway known as the type III secretion system (T3SS). The T3SS is encoded by numerous Gram-negative pathogens and enables these bacteria to secrete and inject pathogenic effector molecules into the cytosol of eukaryotic host cells. About 20 gene products, most of which are inner-membrane proteins, comprise the T3SS. The components mediating type III secretion are conserved in pathogens as diverse as *Yersinia* and *Erwinia*, but the secreted effector proteins vary significantly between species. The T3SS apparatus, which appears to span the periplasmic space, resembles the basal body of a flagellum connected to a straight rod that extends across the outer membrane. Interestingly, all T3SS encode some components with homologies to proteins involved in flagellar assembly. The secretion of proteins by the T3SS is an ATP-dependent process that involves no distinct periplasmic intermediates. Type III-secreted proteins of EPEC, the nodule-forming strains of *Rhizobium* species and *Sinorhizobium fredii*, and several plant pathogens including *Pseudomonas syringae* pathovar tomato, *Erwinia amylovora, R. solanacearum*, and *Xanthomonas campestris* have been shown to assemble into pilus-like structures.

EPEC encodes four proteins, EspA, EspB, EspD, and Tir, which are secreted by a type III pathway. These proteins facilitate intimate contact between the pathogen and host intestinal cells and are required for the formation of specific (attaching and effacing) lesions. In 1998, Knutton and colleagues showed that one of these proteins, EspA, can assemble into 10- to 12-nm-thick peritrichously expressed pilus-like fibers that are organized into ∼50-nm-wide bundles and extend up to 2 μm from the bacterial surface (**Figures 10(a)** and **10(b)**). The assembly of these EspA filaments requires several other components of the T3SS, including an outer-membrane secretin called EscC, a type III translocator protein EspD, the T3SS ATPase EscN, and EscF, the major structural protein of the T3SS needle complex. Coiled-coil interactions of EspA polypeptides lead to the assembly of EspA filaments in a process similar to that of flagellar assembly from flagellin subunits. During the infection process, the EspA fibers appear to mediate contact between EPEC and the host cell surface before the establishment of more intimate bacterial attachment. The EspA fibers seem to assist the translocation of EspB and other effector molecules into host cells where they can subvert host signal transduction pathways.

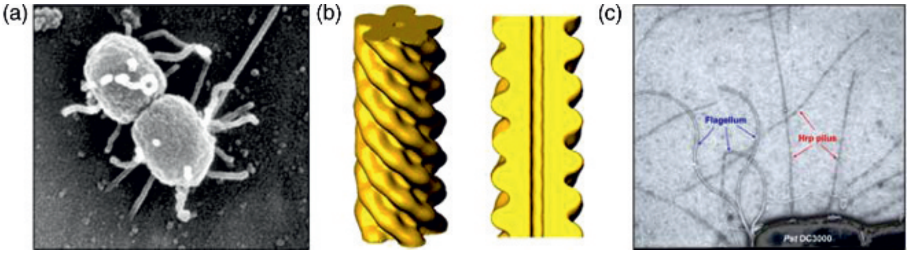

Figure 10 Pili assembled by a type III secretion pathway. (a) Scanning electron micrograph of EPEC elaborating ~50-nm-thick bundles of pili containing EspA, a protein exported by a type III secretion pathway. The individual 6- to 8-nm-thick pili comprising the bundles are not resolved in this micrograph. Reproduced from Knutton S, Rosenshine I, Pallen MJ, Nisan I, Neves BC, Bain C, Wolff C, Dougan G, and Frankel G (1998). *The EMBO Journal* 17: 2166–2176, with permission from Oxford University Press. (b) Three-dimensional reconstruction of EspA filaments showing a tilted side view (left) and cut-away side view (right) revealing a central channel. Reproduced from Daniell SJ, Kocsis E, Morris E, Knutton S, Booy FP, and Frankel G (2003). *MolecularMicrobiology* 49: 301–308, with permission from Blackwell Publishing. (c) Electron micrographs of negatively stained *Pseudomonas syringae* expressing Hrp pili (about 8 nm in diameter) and flagella (about 15 nm in diameter). Photo provided by S. Y. He. Reproduced from He SY and Jin Q (2003). *Current Opinion in Microbiology* 6: 15–19), with permission from Elsiever Ltd.

In *P. syringae* and other plant pathogens, T3SS are encoded by *Hrp* and *Hcp* (hrp conserved) genes. *Hrp* stands for hypersensitive response and pathogenicity, reflective of these genes' roles in causing disease in susceptible plants and eliciting the hypersensitive response (a rapid, localized host cell death, which limits the spread of a pathogen) in resistant plants. HrpA from *P. syringae* and *E. amylovora*, HrpE from *X. campestris*, and HrpY from *R. solanacearum* have all been shown to be assembled into 6- to 8-nm-wide, 2-μm-long peritrichously expressed pili (**Figure 10(c)**). These Hrp pili subunits are small proteins (6–11 kDa) with significant variation in their amino acid sequences, but their assembled pili are remarkably consistent α-helix-rich structures. Assembly of Hrp pili contrasts against all other known pilus assembly systems in that subunits are added at the tip, rather than at the base, of the pilus. The Hrp secretion system is always found at Hrp pilus assembly sites, suggesting that the pili act as a conduit or guiding filament for the delivery of effector proteins into host cells. Hrp pili might also be in involved in the penetration of the host plant cell wall, perhaps by pore formation or cell wall modification during effector delivery.

Pili in Gram-Positive Bacteria

Pili have been observed on a number of Gram-positive bacteria, but to date their study is less advanced than that of their Gram-negative counterparts. The best-defined Gram-positive pili are made by *Corynebacterium diphtheriae*, which carries at least three different types, designated SpaA, D, and H after their major subunits. SpaA is considered the prototypical Gram-positive pilus type, and most of the following information (and the model for pilus assembly in **Figure 11**) is based on findings from *C. diphtheriae* SpaA. It should be noted however that other *Corynebacterium* spp., *Actinomyces* spp., and *Streptococcus* spp. are believed to form pili in a similar manner. In addition, Gram-positive species *Arthrobacter photogonimos* and *Ruminococcus albus* are believed to assemble pili by a separate mechanism(s) that has not yet been elucidated.

Pili on Gram-positive bacteria differ from Gram-negative pili in several important ways. Most notably, while the subunits of Gram-negative pili are held together by noncovalent attachments, Gram-positive pilin subunits attach to each other, and to cell wall peptidoglycan, by covalent bonds. Sortases – transpeptidases named for their role in 'sorting' proteins to the cell wall fraction – are required for the formation of these covalent bonds. The SpaA, D, and H pili in *C. diphtheriae* are named for this requirement, where spa stands for sortase-mediated pilus assembly.

Like many Gram-negative pilins, Gram-positive pilus subunits carry N-terminal signal peptide sequences that target them for secretion through the cell membrane by the *sec* machinery. Spa-like pilins also carry crucial residues at the C-terminus: a cell wall-sorting pentapaptide (usually LPXTG), a hydrophobic region of 30–40 amino acids, and a positively charged tail. Upon translocation by the *sec* machinery, the pilin's hydrophobic region is inserted into the membrane, and held in place by the charged tail. Membrane-associated sortases then cleave the subunit's recognition peptide, leaving the C-terminus embedded in the membrane while the body of the pilin is attached to the enzyme to form an acyl-enzyme intermediate. Nucleophilic attack by an amino group – on either a peptidoglycan precursor (for joining to the cell wall) or another pilin subunit (for pilus growth) – releases the pilin from the sortase enzyme. This process is depicted in **Figure 11**.

Spa pili in *C. diphtheriae* are thin fiber-like structures, 2–6 nm in diameter and 0.2–3 μm long. Each pilus is composed primarily of its major subunit, although some also include a tip subunit and other minor subunits along the shaft that are not required for pilus synthesis or integrity. Major pilus subunits contain a conserved pilin

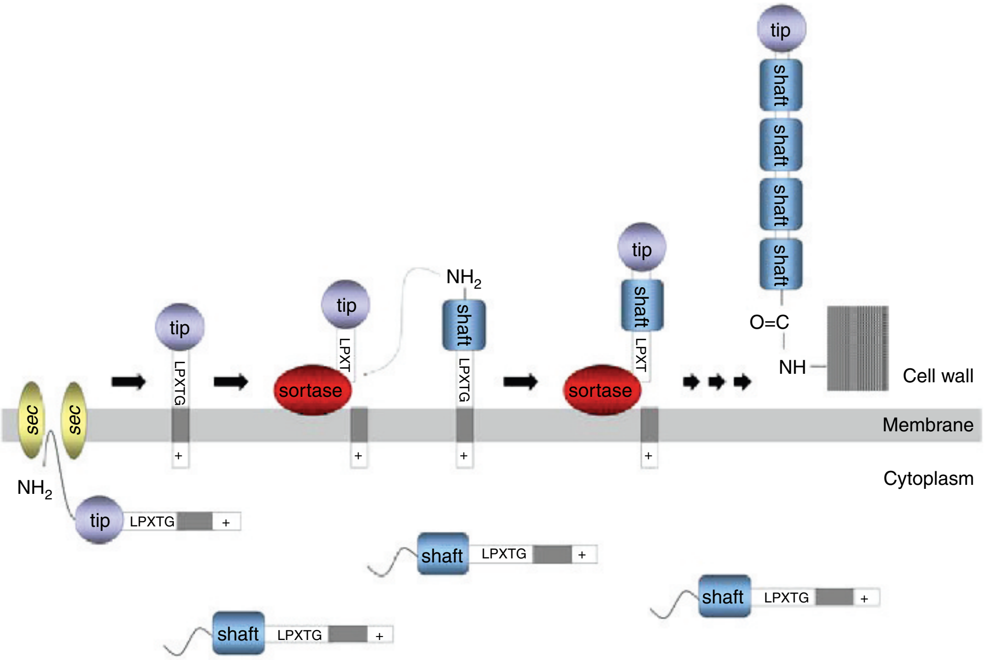

Figure 11 Generalized model of pilus assembly in Gram-positive bacteria. SpaA pili are composed of three types of pilins: SpaA, which forms most of the pilus shaft; SpaB, a minor subunit of unknown function not included in this schematic diagram; and SpaC, which is found at the pilus tip. Other Spa-like pili have similar components. The tip protein is probably the first pilin to be incorporated into a new pilus. Upon translocation by the *sec* machinery (mediated by an N-terminal secretion signal), a pilin's hydrophobic region is inserted into the membrane and held in place by a positively charged tail. A membrane-associated sortase then cleaves the subunit's recognition peptide (usually LPXTG), leaving the C-terminus embedded in the membrane while the main body of the pilin is attached to the enzyme to form an acyl-enzyme intermediate. Nucleophilic attack by an amino group on another pilin forms a peptide bond between subunits, leading to growth of the nascent pilus. The pilus is covalently attached to the cell wall through nucleophilic attack by an amino group on a peptidoglycan precursor, probably via a separate housekeeping sortase. Reproduced from Ton-That H and Schneewind O (2004). *Trends in Microbiology* 12: 228–234, with permission from Elsiever Ltd.

motif, which is believed to mediate release of the previous pilin from its sortase by nucleophilic attack. The absence of this motif in tip subunit SpaC may account for its tip-only localization, as it is able to attach to another subunit at only one site rather than two. Minor subunit SpaB also lacks the pilin motif, but is found intermittently along the length of the pilus. The role and mechanism of incorporation for minor subunit SpaB have not yet been defined.

Each of the three Spa pilus types in *C. diphtheriae* has its own gene cluster, encoding one major subunit, two minor subunits, and one or two sortase-like transpeptidases. *C. diphtheriae* encodes six sortase (srt) homologues in all, five of which are within predicted pilus gene clusters. *SrtA*, which is adjacent to *SpaABC*, is required for assembly of SpaA pili. The SpaD pilus gene cluster includes both SrtB and SrtC, and either of these sortases is sufficient for SpaD pilus assembly. SrtF, which is not linked to a pilus, is likely a 'housekeeping' sortase, responsible for anchoring a variety of LPXTG motif-containing proteins in the cell wall. SrtF is also required for firm attachment of SpaA pili, indicating that its cell wall-anchoring activity may be important for pilins as well as for nonpilin proteins. Because SrtA seems to have limited efficiency in attaching pilins to the cell wall, it has been argued that the term sortase is a misnomer, and that SrtA should instead be referred to as a pilin polymerase. Further research will be required to establish the exact roles of the sortase-like transpeptidases in pilus assembly and attachment.

As in Gram-negative bacteria, pili play a vital role in mediating attachment of Gram-positive bacteria to target surfaces and tissues, and thus also in pathogenesis. The dental pathogen *Actinomyces naeslundii* uses two different types of pili: one promotes colonization by adhering to saliva-coated tooth enamel and the other mediates attachment and interactions with both mammalian cells and other bacteria, potentially promoting both infection and biofilm formation. *S. pneumoniae* lacking pili show impaired adherence to lung epithelial cells, and are less virulent than their wild-type counterparts in direct competition. Recent work in clinically important group A and group B *Streptococcus* strains has shown that pili can be effective vaccine candidates for Gram-positive pathogens. Given these clinical implications, one may expect an increase in research on Gram-positive bacterial pili in the coming years.

Regulation of Pilus Biogenesis

Pilus biogenesis, in general, is a tightly regulated process. Ideally, the costs in energy and other resources required for pilus assembly must be balanced with potential benefits that pilus expression might provide a bacterium. For example, by producing pili in a nutritionally poor environment, a bacterium will tax its available resources, but with pili the same bacterium may be able to gain access to a more favorable location. Pathogenic and other bacteria must also control pilus expression, in some cases, to avoid attachment to unfavorable sites (tissues) within their hosts. Furthermore, pathogenic bacteria may need to modulate pilus expression to escape detection by the host immune system. Whether or not a bacterium expresses pili is greatly affected by environmental factors. Changes in temperature, osmolarity, pH, oxygen tension, carbon source, and nutrient availability may either increase or decrease pilus expression. The presence of iron, aliphatic amino acids, and electron acceptors other than oxygen may also influence the expression of pili. A combination of these environmental cues can stimulate (or repress) pilus synthesis and alter the expression of a number of other factors, all of which can influence the tropism of bacteria for specific niches within the environment or within host organisms.

Environmental signals affect pilus biogenesis through global regulator proteins that can modify the transcription of pilus genes. Various global regulators have been identified and include H-NS, a DNA-binding histone-like protein that often mediates temperature regulation of pilus synthesis. H-NS appears to alter DNA topology and typically functions as a negative regulator. Regulation by carbon source can occur through the catabolite activator protein (CAP), whereas the leucine-responsive regulatory protein (Lrp) can modulate pilus expression in response to aliphatic amino acids. The CAP and Lrp regulators can control sets of pilus operons, enabling the expression of different types of pili to be coordinated and integrated with the metabolic state of the bacterial cells. In addition to these and other global regulators, specific regulator proteins encoded by genes within some pilus operons may also modulate pilus biogenesis. Multiple regulatory factors can act upon the same promoter region, switching pilus gene expression from on to off and vice versa. This on and off switching, known as phase variation, can also be modulated by the methylation status of a promoter region and by the inversion of sequence elements within a promoter. Extracytoplasmic and cytoplasmic stress response pathways also appear to be involved in regulation of the assembly and function of a large number of different pilus types. In keeping with the vast array of niches filled by bacteria and the different environments to which they must adapt, regulation of pilus biogenesis is highly complex, and may vary widely among strains.

Role of Pili in Disease Processes

The expression of pili can have substantial impact on the establishment and persistence of pathogenic bacteria within both plant and animal hosts. For many bacterial pathogens, adhesive pili play a key role in the colonization of host tissues. Uropathogenic *E. coli*, for example, require type 1 to effectively colonize the bladder epithelium. These pili attach to conserved, mannose-containing host receptors expressed by the bladder epithelium and help prevent the bacteria from being washed from the body with the flow of urine. Type 1 also mediate invasion of bladder cells, and can promote bacterial survival by modulating the host immune response. For example, P pili may serve a similar function in the kidneys, preventing the clearance of pyelonephritic *E. coli* from the upper urinary tract and allowing the establishment of an infection. Enteric pathogens produce a wide variety of adhesive pili that facilitate bacterial colonization of the intestinal tract. These include the K88, K99, and 987P pili made by ETEC strains, the long polar fimbria and plasmid-encoded fimbria of *S. enterica* ser. Typhimurium, the subclass of Afa/Dr adhesins, including F1845, Dr and Afa-3 fimbriae of *E. coli*, and aggregative adherence fimbria (AAF) of enteroaggregative *E. coli.* In the small intestine, toxin coregulated pili (TCP) are essential for the attachment of *V. cholera* to gut epithelial cells. These pili also act as receptors for the cholera toxin phage (CTXΦ), a lysogenic phage that encodes the two subunits of the cholera toxin. This phage, with its encoded toxin, is transferred between *V. cholera* strains via interactions with TCP within the small intestine. Other pili also function in the acquisition of virulence factors. The uptake of DNA facilitated by type IV pili and DNA transfer directed by conjugative pili can provide pathogens with accessory genes, enabling them to synthesize a wider repertoire of virulence factors and giving them resistance to a greater number of antibiotics. Biofilm formation, which in some cases appears to require pili such as type 1, type IV, or curli, can also increase the resistance of bacteria to antibiotic treatments and may aid bacterial colonization of tissues and medical implants.

Pili are not necessarily static organelles, and dynamic alterations of pilus structures during the infection process may influence the pathogenicity of piliated bacteria. For example, electron microscopic studies of mouse bladders infected with type 1-piliated uropathogenic *E. coli* showed that the pili mediating bacterial adherence to the bladder epithelial cells were 10–20 times shorter than typical type 1. It is possible that the shorter type 1 observed are the result of pilus retraction, compact coiling, or breakage during the infection process. The shortening of pili may provide a means for reeling bacteria in toward their target host cells, allowing the bacteria to make intimate contact with the host after initial attachment at a distance. Within the gut, type IVb pili (BFP) promote autoaggregation and microcolony formation in EPEC strains, a phenomenon that facilitates the adherence of EPEC to the intestinal epithelium. After initial attachment, an energy-dependent conformational change in the quaternary structure of BFP appears to be needed for the further dispersal of EPEC over human intestinal cells and for the full virulence of this pathogen.

During the infection process, adhesive pili are often situated at the interface between host and pathogen where they can potentially mediate cross-talk between the two organisms. Pilus attachment to the receptor of a host eukaryotic cell may induce a number of host signal transduction pathways, potentially leading to cytoskeleton rearrangements, membrane ruffling, and pathogen internalization. For example, binding of the type IVa pili of *Neisseria* to host receptors (probably CD46) on target epithelial cells has been shown to stimulate the release of intracellular Ca^{2+} stores, a signal known to control a multitude of eukaryotic cellular responses. Likewise, the attachment of P pili to Galα(1–4)Gal-containing host receptors on target uroepithelial cells can trigger the intracellular release of ceramides, important second messenger molecules that are capable of activating a variety of protein kinases and phosphatases involved in signal transduction processes. The signals induced within uroepithelial cells on binding of P-piliated bacteria eventually result in the secretion of several immunoregulatory cytokines. Binding of type 1-piliated bacteria to mannosylated receptors on uroepithelial cells can similarly induce the release of cytokines, although apparently through different signaling pathways than those stimulated by P pilus binding. It has also been suggested that pili can transduce signals into bacterial cell, although this reported phenomenon requires further investigation and verification. Continued research into the biogenesis, structure, and function of pili not only promises to advance our basic understanding of the role of these organelles in pathogenic processes but may also aid the development of new-generation antimicrobial therapeutics and vaccines. Indeed, over the last decade, a tremendous amount of effort has been directed toward the development of pili-based vaccines and small molecule inhibitors called pilicides that can interfere with pilus assembly. At a time when the number of antibiotic-resistant bacterial strains is on the rise, effective pili-based vaccines and pilicides promise to be especially useful tools in combating bacterial infections.

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Plant Pathogens and Disease: General Introduction

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Defining Statement
Introduction: Causes of Disease in Plants
Pathogens that Cause Disease in Plants
Interactions of Pathogens, Plants, and Humans
Conclusion
Further Reading

Glossary

disease cycle The chain of events involved in the life cycle of a pathogen and in the development of the disease caused by this pathogen.

effectors Proteins produced by pathogens that act to suppress the defenses of the plant basal immune system but in some cases trigger the defenses of the host plant.

elicitor A molecule coded by a gene for avirulence of a pathogen, which, upon contact with the receptor molecule of the corresponding plant gene for resistance, triggers the defense reaction of the plant that keeps it resistant to the pathogen.

haustoria Feeding organs of some fungi and parasitic higher plants that enter the host tissues and absorb nutrients from host cells.

life cycle The stages in the growth and development of an organism that occur between two successive occurrences of the same stage, for example, sporulation of the organism.

monopartite A virus whose each particle contains the total nucleic acid of the virus.

parenchyma Plant tissue consisting of thin-walled cells with intercellular spaces between them and synthesize or store foodstuffs.

phloem Tubelike cells of the plant conductive system that carry sugar and other organic molecules from leaves to other parts of the plant.

sporulate Produce spores.

transgenic An individual that has been transformed with and carries genes obtained from other organisms and are expressed by these organisms.

vector A specific insect, fungus, nematode, and so on, that can acquire and transmit a specific pathogen from an infected to a healthy host plant.

virulence Relative ability of a pathogen to cause disease on a given host plant.

Abbreviations

DPM	Doctor of Plant Medicine
HR	hypersensitive response
TMV	tobacco mosaic virus

Defining Statement

Plant pathogens are mostly microorganisms, such as bacteria, viruses, fungi, nematodes, and protozoa, and some parasitic plants and algae. They attack plants, obtain nutrients, and cause disease by releasing enzymes, toxins, and so on. Successful infections depend on the interaction of appropriate genes present in pathogens and in plants. Plant diseases cause financial losses, hunger and famines, food poisoning, and extinction of plant species.

Introduction: Causes of Disease in Plants

Plants, like humans and animals, are injured or damaged by abiotic, that is, environmental, factor, and by biotic ones, such as various pests, for example, insects, and by diseases caused by pathogens (**Figure 1**). In all organisms, diseases and injury are caused by internal or external abiotic, that is, environmental factors, such as nutritional deficiencies, freezing temperatures, droughts, floods, pollution, and so on. (**Figure 2**), or by pathogens. Although abiotic factors may cause damage to plants over extensive areas, they differ from pathogens in that they (1) do not multiply and (2) do not spread from plant to plant. As a result, abiotic factors, although they cause damage to plants, do so much less frequently than plant pathogens, they are unpredictable and, usually, are easy to diagnose but difficult to control.

Pathogens that Cause Disease in Plants

Pathogens are living entities, mostly certain microorganisms, such as certain fungi, prokaryotes such as bacteria and

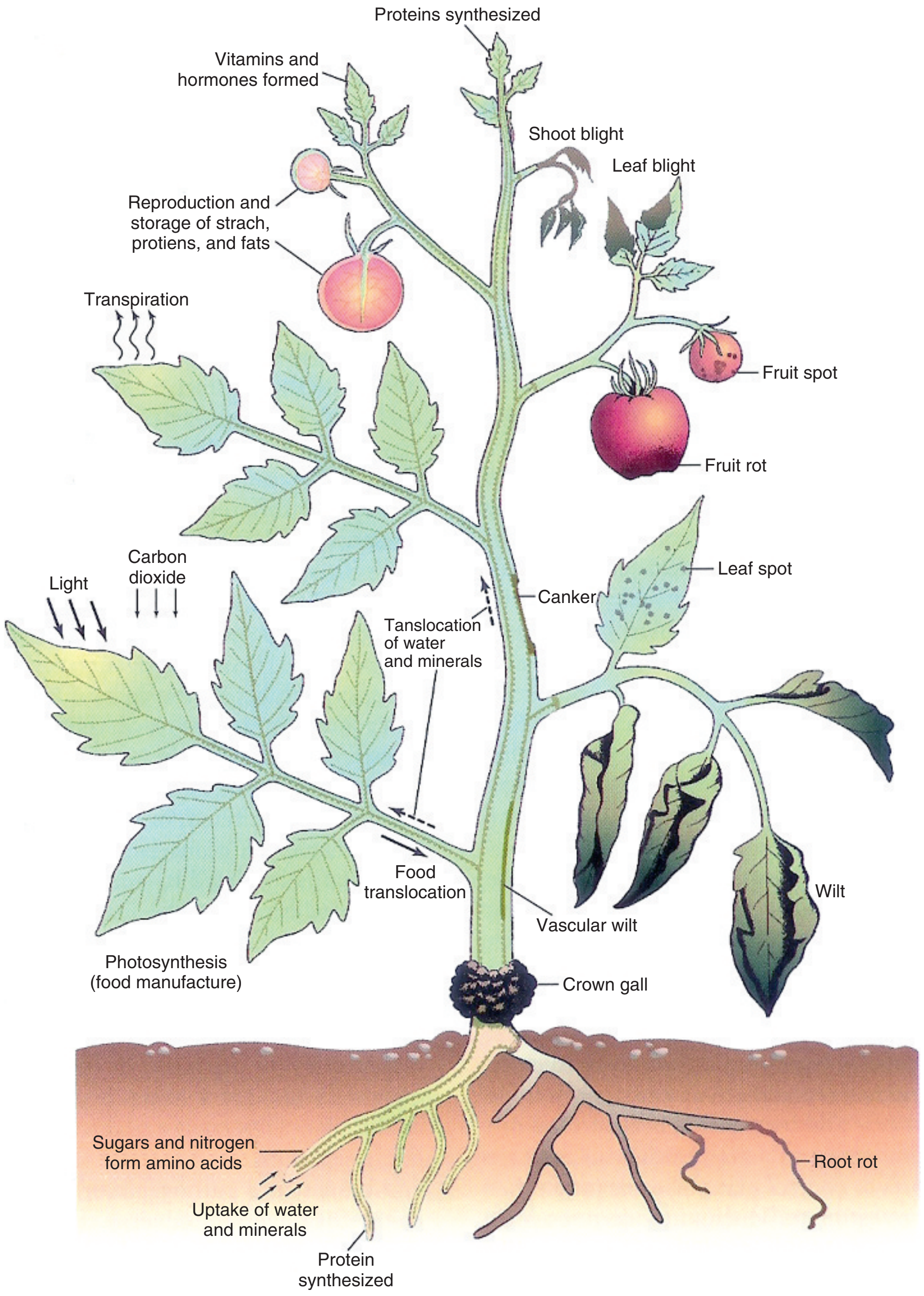
Proteins synthesized
Vitamins and hormones formed
Shoot blight
Leaf blight
Reproduction and storage of strach, protiens, and fats
Transpiration
Fruit spot
Fruit rot
Leaf spot
Carbon dioxide
Light
Canker
Tanslocation of water and minerals
Food translocation
Wilt
Vascular wilt
Photosynthesis (food manufacture)
Crown gall
Sugars and nitrogen form amino acids
Root rot
Uptake of water and minerals
Protein synthesized

Figure 1 Typical plant of which the left half is showing the basic functions of its main organs, whereas the right half is showing the various symptoms of infection by pathogens and their interference with the basic functions. Modified from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 6. Burlington, MA: Elsevier/Academic Press.

mollicutes, viruses, protozoa, nematodes, and so on, which can attack other organisms and cause disease. In addition to these pathogens, diseases in plants can also be caused by several plants that parasitize other plants, and by some parasitic green algae. However, parasitic plants and parasitic algae cause only a few important diseases to plants. Furthermore, many pests, such as aphids, mites, and other microorganisms, cause injury in plants closely resembling disease, as does the pressure for food and water, and for space, brought on by numerous weeds. The vast majority of diseases in plants are caused by the same groups of pathogenic microorganisms as those that cause disease in animals and humans.

Plant pathogens vary considerably in size (**Figures 3** and **4**), shape, and method of multiplication (**Figure 4**).

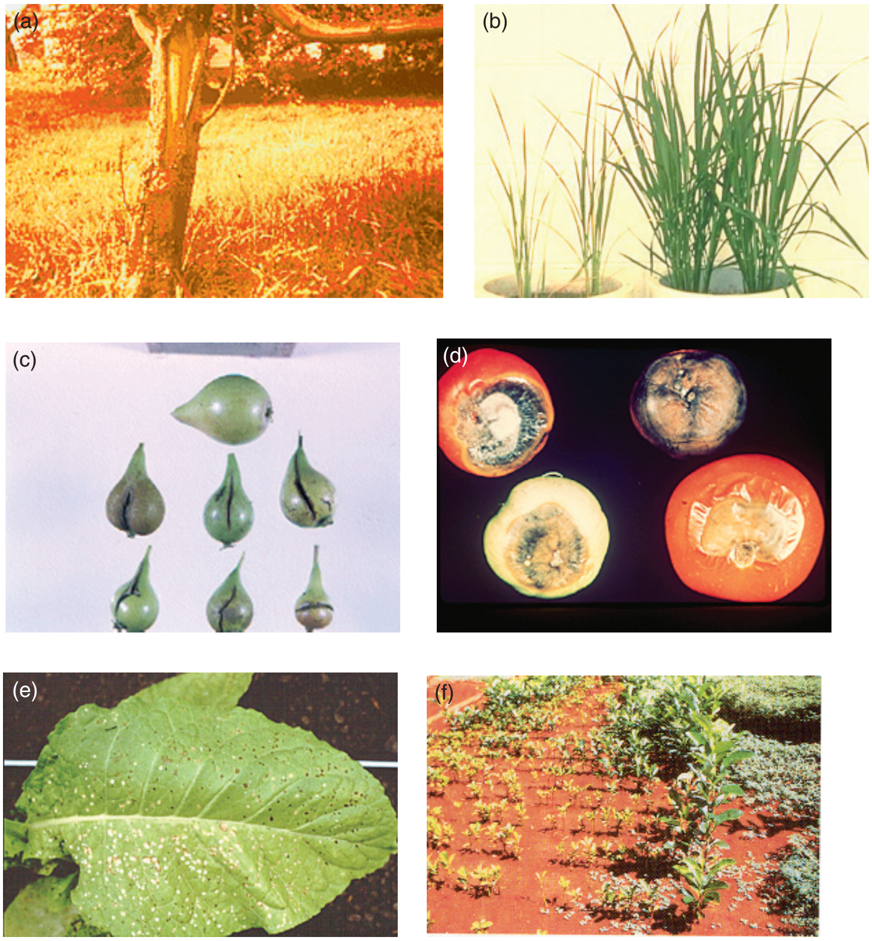

Figure 2 Symptoms of plants and plant organs showing the effect of abiotic factors on plants. (a) Effect of freezing temperatures on the trunk of apple tree. (b) Young barley plants show yellowing and dieback of leaf tips caused by nitrogen (N) deficiency. (c) Young pear fruit showing symptoms of boron deficiency. (d) Tomato fruit showing symptoms of calcium deficiency. (e) Tobacco leaf showing typical symptoms of ozone injury. (f). Citrus seedlings showing yellowing, stunting, and some have been killed by improper fumigation with pesticides. Photos: (a), (b), and (e) courtesy of USDA; (d), Clemson University; (f), JH Graham, University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 362, (b) p. 374, (c) p. 376, (e) p. 370, (f) p. 319.

Like all pathogens, those affecting plants vary considerably in host specificity. Some pathogens are able to infect all or most plants belonging to one species, to a genus, or to several plant families, whereas other pathogens can infect only one or a few varieties within one plant species.

Plant pathogens cause disease in plants by entering and invading plant tissues and by absorbing food stuffs from the host cells for their own growth, multiplication, and spread. By entering the plant, pathogens disturb the structural integrity and the metabolism of plant cells and tissues through enzymes, toxins, growth regulators, and other active substances they secrete (**Figure 5**). Some pathogens may also cause disease by growing and multiplying in the xylem and phloem vessels of plants, thereby blocking the upward movement of water and minerals and the downward translocation of sugars (**Figures 1** and **5**).

Plants infected with pathogens develop a variety of symptoms, such as necrotic spots on leaves, stems, fruit, and roots; blights, that is, the sudden death of leaves and young shoots; cankers, that is, necrotic patches of bark and under-the-bark tissues of branches and trunks; vascular wilts due to blockage by the pathogen of xylem vessels; rots of roots, stems, and fruit; galls on stems or roots; proliferation, that is, excessive branching of shoots and roots; mosaics; stunting; decline; and death of branches or entire plants. Symptoms vary considerably depending on the kind of pathogens that cause them, and they may vary in severity, ranging from insignificant to death of the entire plant. It has been estimated that plant pathogens are responsible for a 16% loss of the attainable annual world crop production, estimated at 1.2–1.3 trillion dollars.

Fungi

It is estimated that there are approximately 1.5 million species of fungi on earth, of which 70 000 species are

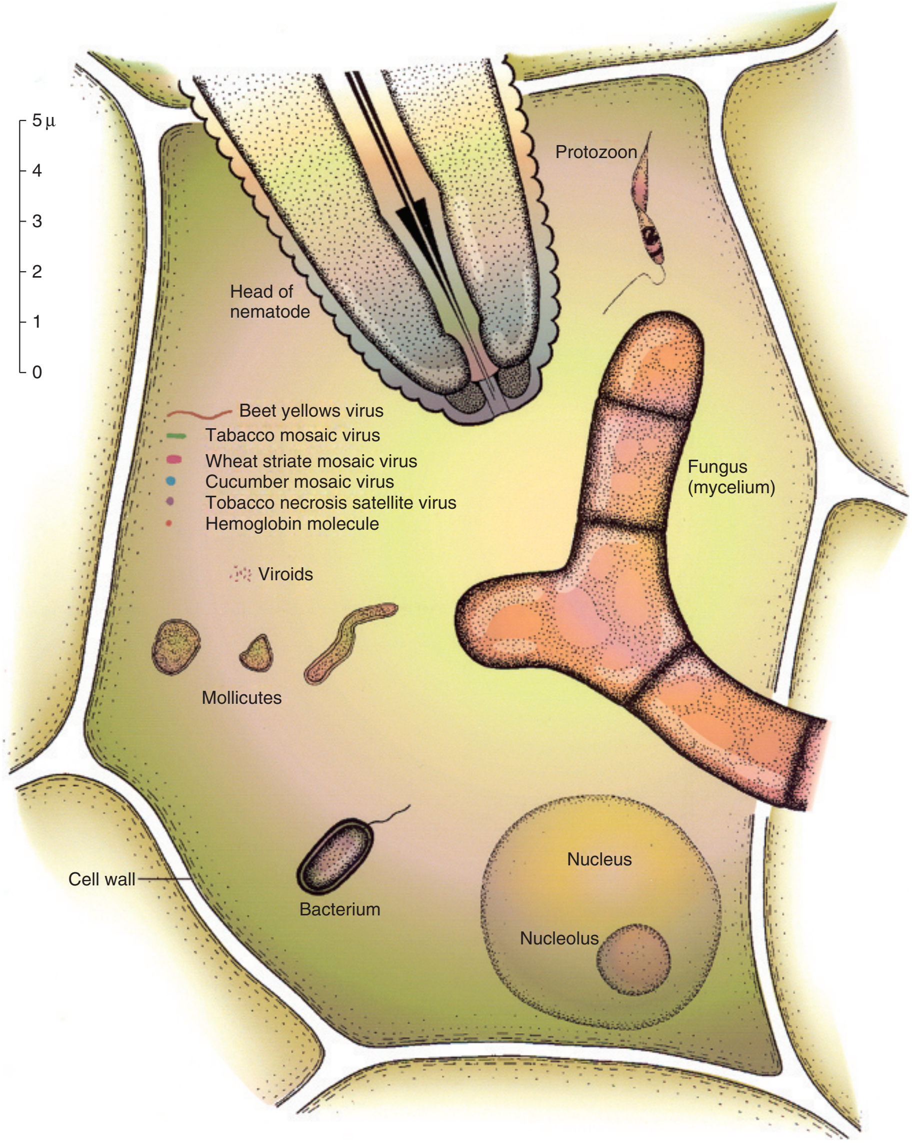
5 μ
4
3
2
1
0
Protozoon
Head of nematode
Beet yellows virus
Tabacco mosaic virus
Wheat striate mosaic virus
Cucumber mosaic virus
Tobacco necrosis satellite virus
Hemoglobin molecule
Viroids
Fungus (mycelium)
Mollicutes
Cell wall
Bacterium
Nucleus
Nucleolus

Figure 3 Morphology and ways of multiplication of some of the groups of plant pathogens. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 8. Burlington, MA: Elsevier/Academic Press.

known and have been studied. Fungi comprise the kingdom Fungi and constitute an independent group of organisms of equal rank to that of plants and animals.

Most pathogens of plants are fungi. They cause the majority (approximately 70%) of all plant diseases. More than 10 000 species of the known 70 000 fungal species can cause disease in plants. Fungi are eukaryotic organisms with one or more chromosomes of DNA contained in well-organized nuclei. Fungal cells, however, contain only one copy of each type of chromosome (haploid), whereas in most eukaryotes the nuclei are diploid, containing two copies of chromosomes. Fungal cell walls contain chitin and glucans.

Some of the plant pathogenic fungi are biotrophs (obligate parasites) because they can grow and multiply only by remaining in constant association with their living host plants. Other pathogenic fungi are nonobligate parasites that either require a living host plant for part of their life cycles, but are able to complete their cycles on dead organic matter, or are able to grow and multiply on dead organic matter (necrotrophs) as well as on living plants.

The vast majority of fungi provide an indispensable service to humanity and the world, by decomposing the vast amounts of plant, especially wood, and animal tissue produced and dying each year. A number of plant pathogenic fungi, such as *Penicillium*, produce penicillin and other antibiotics useful to humans and domestic animals. In addition, several fungi, such as *Trichoderma*, species of *Aspergillus*, and so on, provide various levels of commercially used biological control of soilborne plant pathogenic fungi.

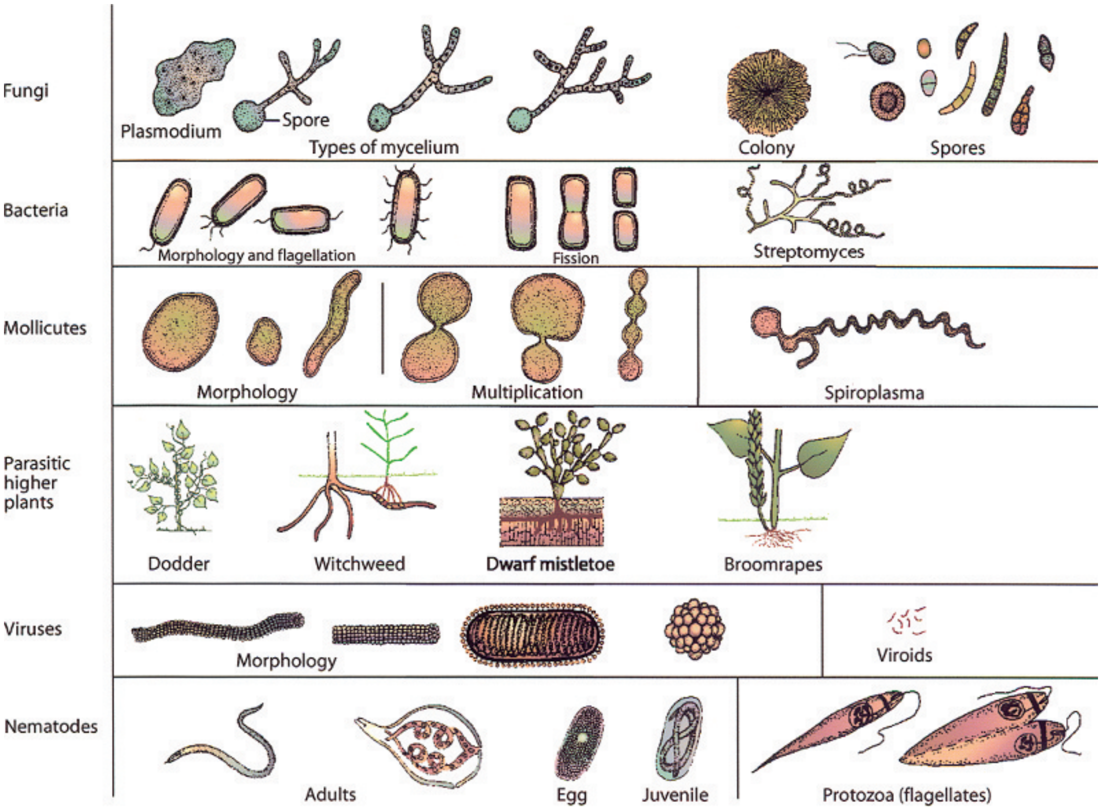
Fungi
Plasmodium
Spore
Types of mycelium
Colony
Spores
Bacteria
Morphology and flagellation
Fission
Streptomyces
Mollicutes
Morphology
Multiplication
Spiroplasma
Parasitic higher plants
Dodder
Witchweed
Dwarf mistletoe
Broomrapes
Viruses
Morphology
Viroids
Nematodes
Adults
Egg
Juvenile
Protozoa (flagellates)

Figure 4 Schematic diagram, for comparison purposes only, of the shapes and sizes of certain plant pathogens in relation to a plant cell. Note: Bacteria, mollicutes, and protozoa are not found in nucleated, living plant cells. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 7. Burlington, MA: Elsevier/Academic Press.

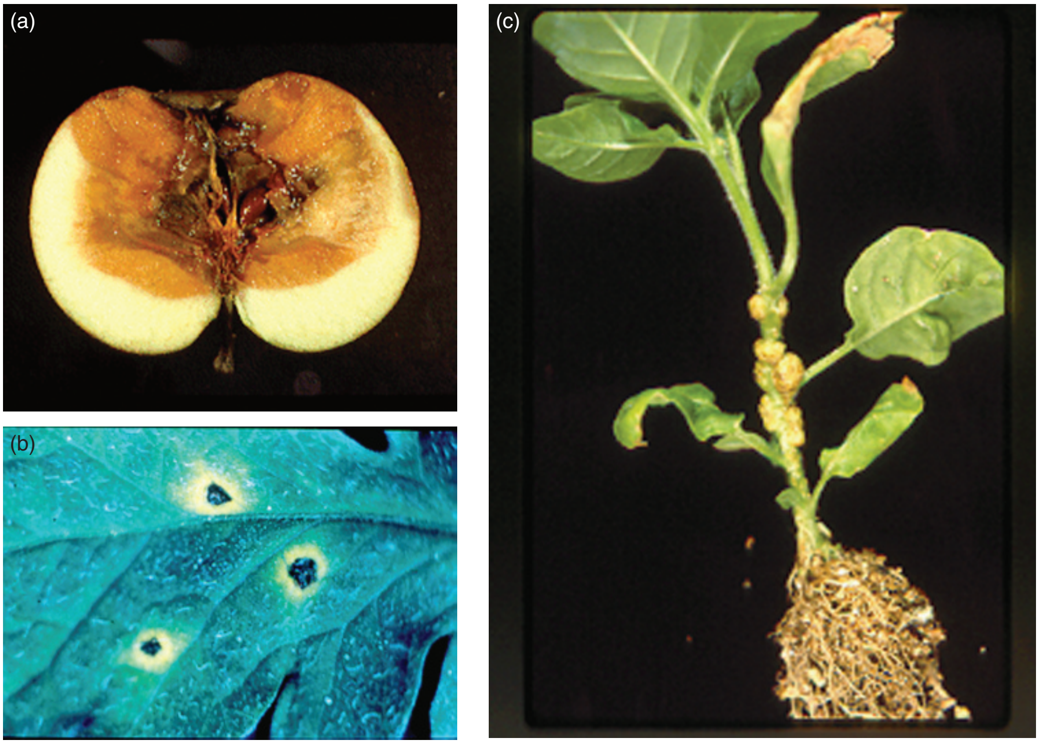

Figure 5 Mechanisms that pathogens use to attack plants. (a) Certain enzymes, such as pectinases and cellulases, which break down plant tissue. (b) Toxins, which kill plant cells, near or at a distance from the location of the pathogen on or in the plant. (c) Overgrowths or galls, which results in infected cells producing or drawing nutrients used by the pathogen. Photo: (b) courtesy of RJ MacGovern, Department of Plant Pathology, University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) and (b) p. 51, (c) p. 663.

Plant pathogenic fungi

Many plant pathogenic fungi are famous for damaging or eliminating plants from nature. This happened, for example, with chestnut blight disease (**Figures 6(a)–6(c)**), caused by the fungus *Cryphonectria parasitica*, which, within about 20 years from the time (1904) the pathogen was brought to North America, destroyed about 4 billion chestnut trees, and almost completely eliminated and threatened with extinction, the American chestnut in North America. A similar direction of destruction, elimination, and possible extinction of the American elm in North America has been followed by the Dutch elm disease (**Figures 6(d)–6(f)**), caused by the fungus *Ophiostoma nova-ulmi*; near elimination of several species of red oak in the Northeastern United States by the oak wilt fungus, *Ceratocystis fagacearum*; near elimination of several oak species in the Pacific Coast states by the recent outbreak of 'oak sudden death' disease caused by the oomycete *Phytophthora ramorum*; and others. Certain species of some fungi, for example, special forms of *Fusarium lycopersici*, the cause of Fusarium vascular wilt in several crops, for example, *F. oxysporum* f. sp. *cubense*, the cause of banana wilt (Panama disease). Once such pathogens are introduced into a field, they become permanent inhabitants of the field and destroy the plant they infect, in this case, banana, for ever after.

A number of plant pathogenic fungi, such as *Aspergillus*, *Penicillium*, *Claviceps*, *Fusarium*, *Trichoderma*, and so on, produce, in plant seeds infected by these fungi, extremely poisonous toxins, called mycotoxins (**Figure 7**), some of which are the most potent carcinogens known. Every year, a

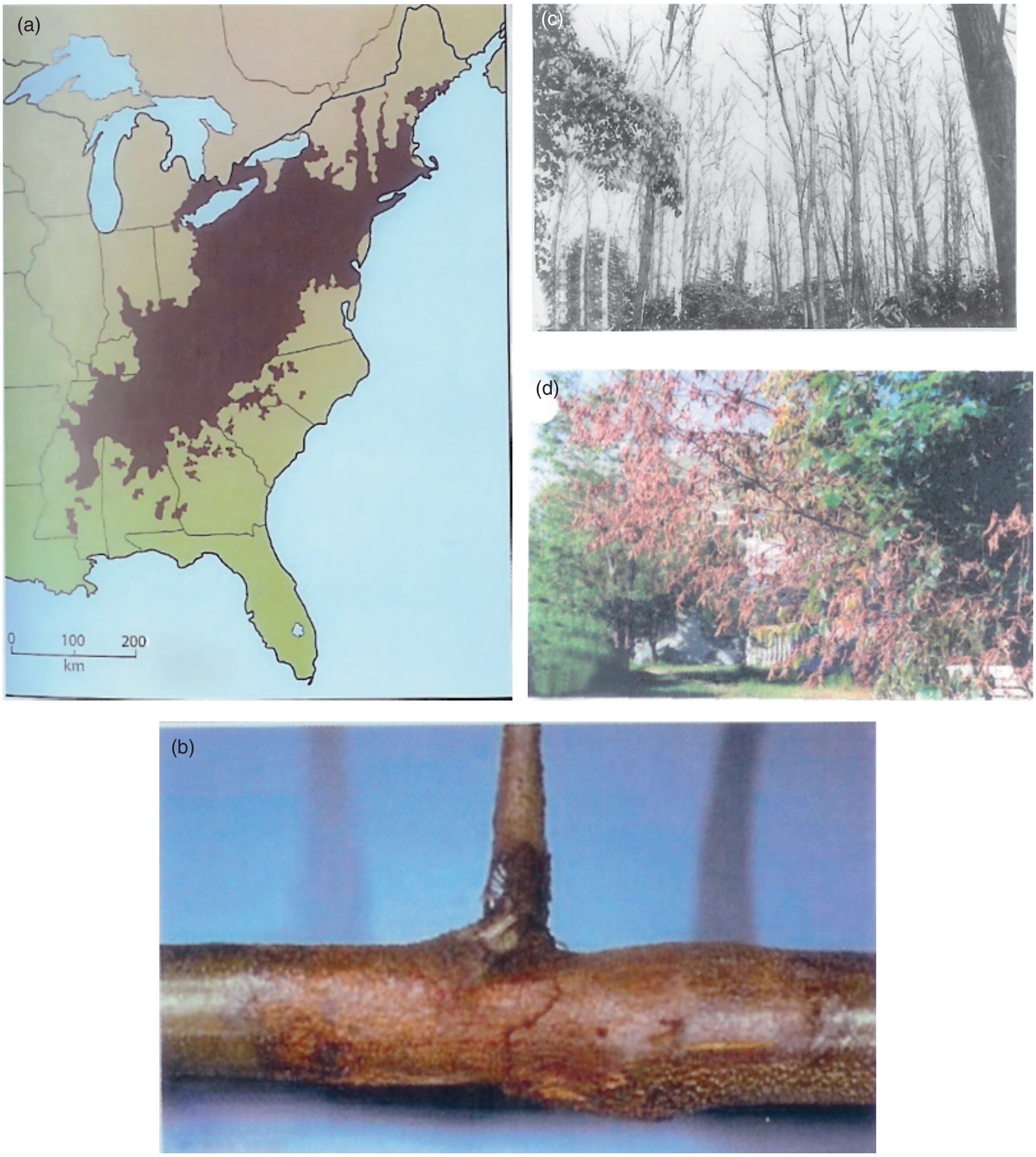

Figure 6 (*Continued*)

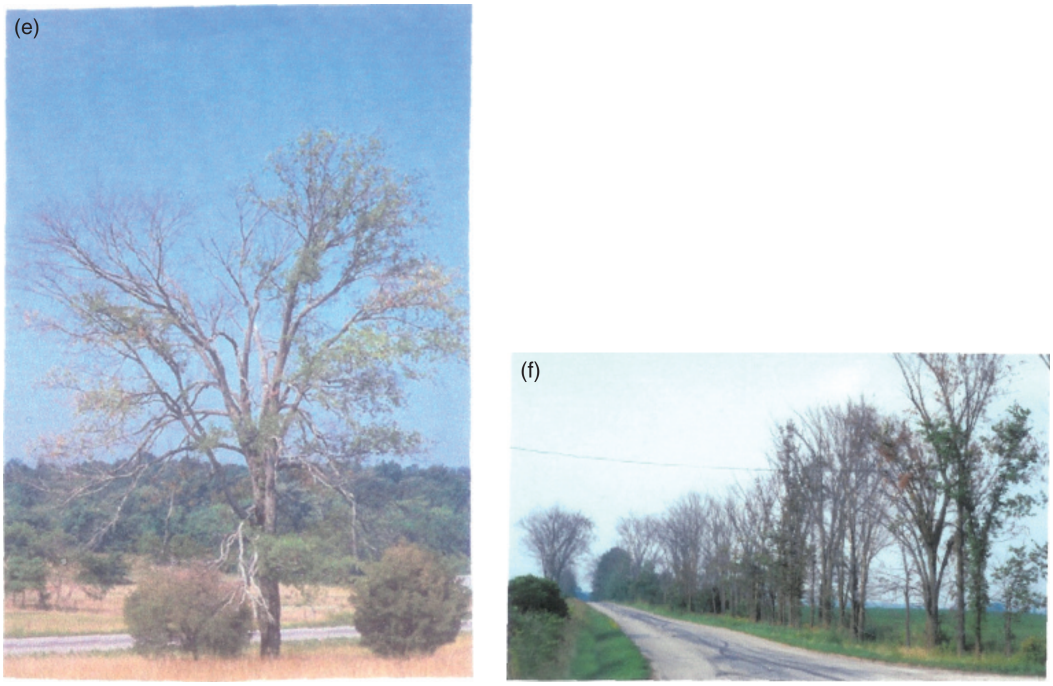

Figure 6 Two catastrophic diseases of trees, chestnut blight (**Figures 6**(**a**)–**6**(**c**)) and Dutch elm disease (**Figures 6**(**d**)–**6**(**f**)), are caused by two different fungi that enter the vascular tissues of the trees and inhibit the passage of water to the tree branches.
(a) Chestnut blight disease, caused by the fungus *Cryphonectria*, has killed nearly all 4 billion American chestnut trees in the plant's natural range (a) with its spores spreading from diseased to healthy tree, infecting branches and the trunks of small trees, causing cankers (b) on tree branches and small trunks, and blocking the passage of water beyond the canker. Multiple cankers on the tree result in the death of the whole trees (c). Photos: (b) courtesy of WL MacDonald, West Virginia University; (c), RL Anderson, US Forest Service. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a), (b), and (c) p. 33.
(b) Dutch elm disease, caused by the fungus *Ophiostoma*, has killed almost all American elm trees along streets of cities and towns in North America and many of those growing in forests. The fungus spores, which spread from tree to tree by two small beetles, grow inside and clog the xylem vessels of twigs and branches, which wilt (d), then the infected branches die (e) and soon after, whole trees die (f). Photos: (d) courtesy of RJ Stipes; (e), R.L. Anderson; (f), EL Barnard, Florida Forest Service. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (d), (e), and (f) p. 34.

number of these adversely affect humans and animals in many parts of the world. On the other hand, many mycorrhizal fungi live symbiotically with the plants they infect and seem to improve the resistance of the host plants to other pathogens, whereas the endophytic fungi *Epichloe* (*Acremonium*) and others, infecting many grasses, are poisonous to animals that consume such grasses (**Figure 7**(**c**)).

Morphology: Shapes and sizes of fungi

Most fungi have a branching filamentous body called mycelium (**Figures 2**, **3**, and **8**). Mycelium produces numerous branches that grow outward in a radial fashion and produces a colony (**Figure 2**). Each branch of the mycelium, called a hypha, is tubular and generally is of uniform thickness (1–5 μm in diameter). In some fungi, the mycelium is more or less a continuous tube containing many nuclei; in others, the mycelium is partitioned into cells by cross-walls, called septa, with each cell containing one or two nuclei. The length of the mycelium in some fungi is only a few millimeters, whereas in others it may be several centimeters long.

Reproduction

Fungi reproduce primarily by means of spores (**Figures 4** and **8**), which may consist of one or a few cells. In different fungi, spores may form asexually, like buds on a twig, or sexually, as the result of a sexual fertilization. Asexual spores in some fungi are produced inside containers called sporangia and such spores are called sporangiospores. In some of these fungi, and in the oomycetes, the sporangiospores have flagella with which they can swim and are therefore called zoosporangiospores or zoospores. In most fungi, the asexual spores are called conidia and are produced by the cutting off of terminal or lateral cells from special hyphae called conidiophores. In some fungi, conidia and condiophores are produced naked on the mycelium, whereas in others they are produced inside thick-walled containers called pycnidia. Sexual reproduction occurs in most but apparently not all fungi. In sexual reproduction, generally, two cells of similar or dissimilar size and appearance fuse to produce a zygote. Depending on the group of fungi, the zygote is produced and undergoes meiosis inside a germinating zygospore (in zygomycetes), an ascus (in ascomycetes), or a basidium

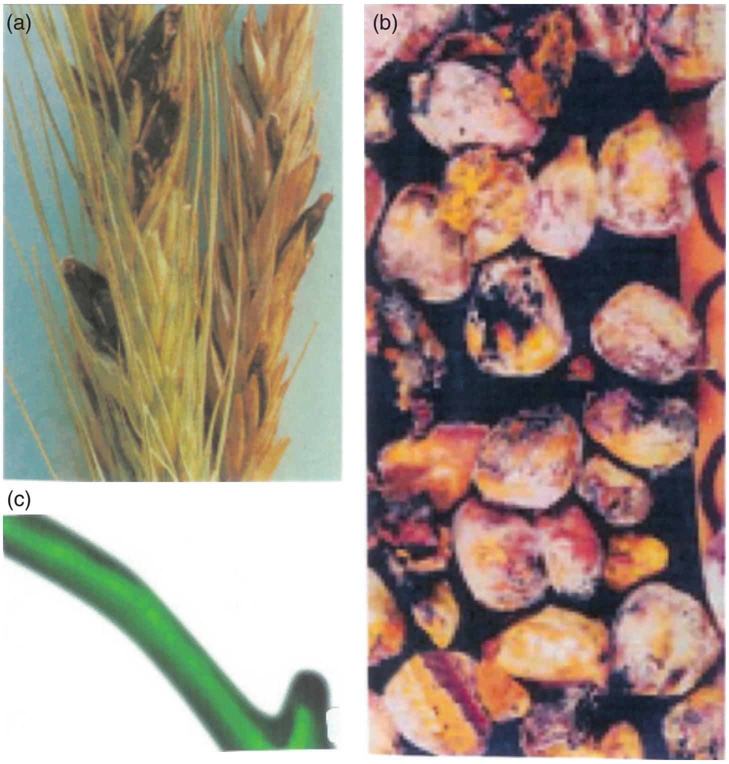

Figure 7 (a) Head of rye plant showing sclerotia (ergots) of the fungus *Claviceps purpurea.* (b) Cracked corn kernels attacked by fungi that produce mycotoxins. (c) Fluorescent mycelium of an endophytic fungus in a grass plant in which it produces mycotoxins. Photos: (a) courtesy of IR Evans, Canada; (b), RW Stack, ND State University; (c), A DeLucca. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 38, (b) and (c) p. 40.

(in basidiomycetes) (**Figure 8**). In some fungi, no sexual spores have been found and these are known as deuteromycetes or imperfect fungi. Some appear to never produce any kind of spores and are known as sterile fungi.

As a result of the different types of mycelium, spores, and spore containers, each group of fungi follows a slightly different life cycle reflecting these differences (**Figures 8** and **9**(**a**)). When the life cycle also incorporates and shows the changes in the host plant, the sequence of events is then called a disease cycle and is much more informative, regarding the interaction of each pathogen with its host, than the life cycle alone. **Figure 9**(**b**) shows a generalized disease cycle applicable to any host–pathogen combination, and lists primarily the events taking place in the fungus and in the plant after the pathogen comes in contact with the host plant. The primary disease cycle after overwintering includes the sexual as well as asexual spores. In contrast, secondary disease cycles develop from pathogens produced from the primary inoculum and usually contain only or mostly asexual spores. **Figure 10** shows the symptoms and most of the stages of the disease cycle of the potato late blight disease caused by the oomycete *Phytophthora infestans*. **Figure 11** shows a fairly detailed disease cycle of the apple scab disease caused by the ascomycete *Venturia inaequalis*, **Figure 12**(**a**) shows the disease cycle, and **Figures 12**(**b**) and **12**(**c**) show the symptoms of stem rust of wheat, caused by the basidiomycete *Puccinia graminis*.

Classification

The fungal pathogens of plants include some microorganisms, the Myxomycota, Plasmodiophoromycota, and Oomycota, that are now known not to be fungi but to belong to different kingdoms of organisms. However, these organisms are similar to fungi and they continue to be studied along with the fungi and the diseases they cause. The following is a sketchy classification of fungal and fungal-like pathogens of plants.

Fungal-like organisms

Kingdom: Protozoa

Phylum: Myxomycota – produce a plasmodium instead of mycelium; they are the surface slime molds. Cause few plant diseases.

Phylum: Plasmodiophoromycota – cause endoparasitic slime mold diseases. Cause a few diseases of importance, for example, clubroot of cabbage.

Kingdom: Chromista

Phylum: Oomycota – produce mycelium that has no cross-walls; their cell walls are composed of cellulose and the amino acid hydroxyproline, not chitin; produce oospores and zoospores; cause many root rots, seedling diseases, foliar

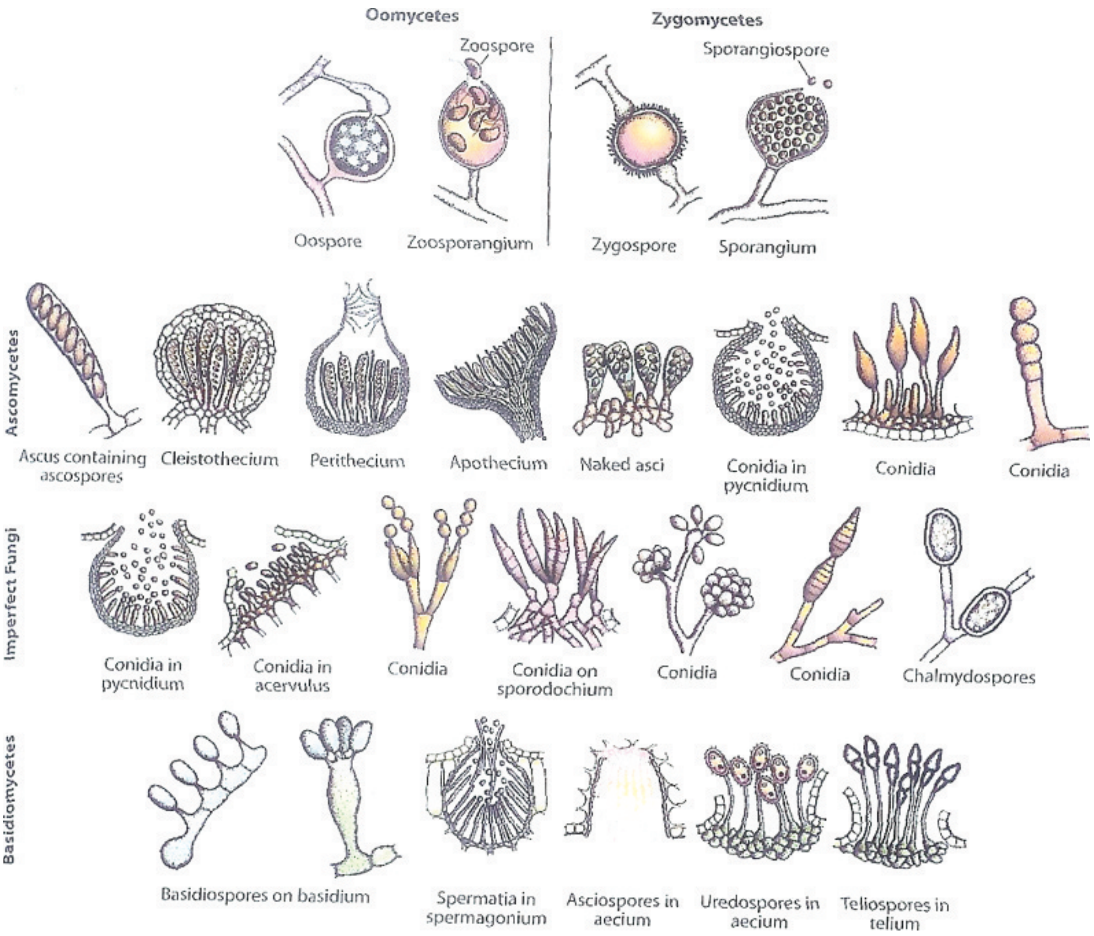
Oomycetes
Zoospore
Oospore
Zoosporangium
Zygomycetes
Sporangiospore
Zygospore
Sporangium
Ascomycetes
Ascus containing ascospores
Cleistothecium
Perithecium
Apothecium
Naked asci
Conidia in pycnidium
Conidia
Conidia
Imperfect Fungi
Conidia in pycnidium
Conidia in acervulus
Conidia
Conidia on sporodochium
Conidia
Conidia
Chalmydospores
Basidiomycetes
Basidiospores on basidium
Spermatia in spermagonium
Asciospores in aecium
Uredospores in aecium
Teliospores in telium

Figure 8 Representative spores and fruiting bodies of the fungal-like oomycetes and of the main groups of fungi. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 389. Burlington, MA: Elsevier/Academic Press.

blights, and the downy mildews. They include the following extremely important pathogens:

Pythium, the cause of many root, stem, and fruit rots.

Phytophthora, the cause of many blights, root and tuber rots, and of cankers, declines, and death of many trees.

Plasmopara, the cause downy mildews.

The true fungi

Kingdom: **Fungi**

Phylum: **Chytridiomycota** – have round or limited elongated nonseptate mycelium, restricted to the host plant, and, alone among the fungi, produce motile zoospores and survive as sporangia. Cause few plant diseases, for example, wart of potato.

Phylum: **Zygomycota** – Order: Mucorales: no zoospores; produce conidia in sporangia; mycelium nonseptate; survive as zygospores; most are saprophytic but a few are weak plant pathogens causing bread molds (**Figure 13**(**b**)) and fruit rots (**Figures 3**(**b**) and **3**(**c**)) in storage.

Order: Glomales: Form vascular – arbuscular mycorrhizae within roots of host plants.

Phylum: **Ascomycota** – Recent, 2007, taxonomic studies have placed most of the 32 000 species of Ascomycetes in the subphylum Pezizomycotina. Under their new umbrella, the species and genera are, of course, similar/identical to Ascomycota, but the Pezizomycotina have septate hyphae, the single septum having a single pore that divides the hyphae into hyphal compartments or cells, and also have Woronin bodies, which are specialized vesicles that seal the septal pore in response to cellular damage. The Woronin body consists of HEX-1 protein that self-assembles and forms the solid form of the vesicle. The Pezizomycotina, like all Ascomycetes, have mycelium that has cross-walls; produce sexual spores (ascospores) within sacs (asci) (e.g., **Figure 7**) that are either naked or contained in fruiting structures of different shapes, namely, cleistothecia, perithecia, and apothecia; produce asexual spores (conidia) on naked hyphae or in containers (pycnidia) or other structures; and they cause the most plant diseases (leaf, stem, and fruit spots and blights, root rots, fruit rots, cankers, vascular wilts, seed rots, etc.).

The new classification scheme rejects the previous taxa of Discomycetes – apothecial fungi, Pyrenomycetes –

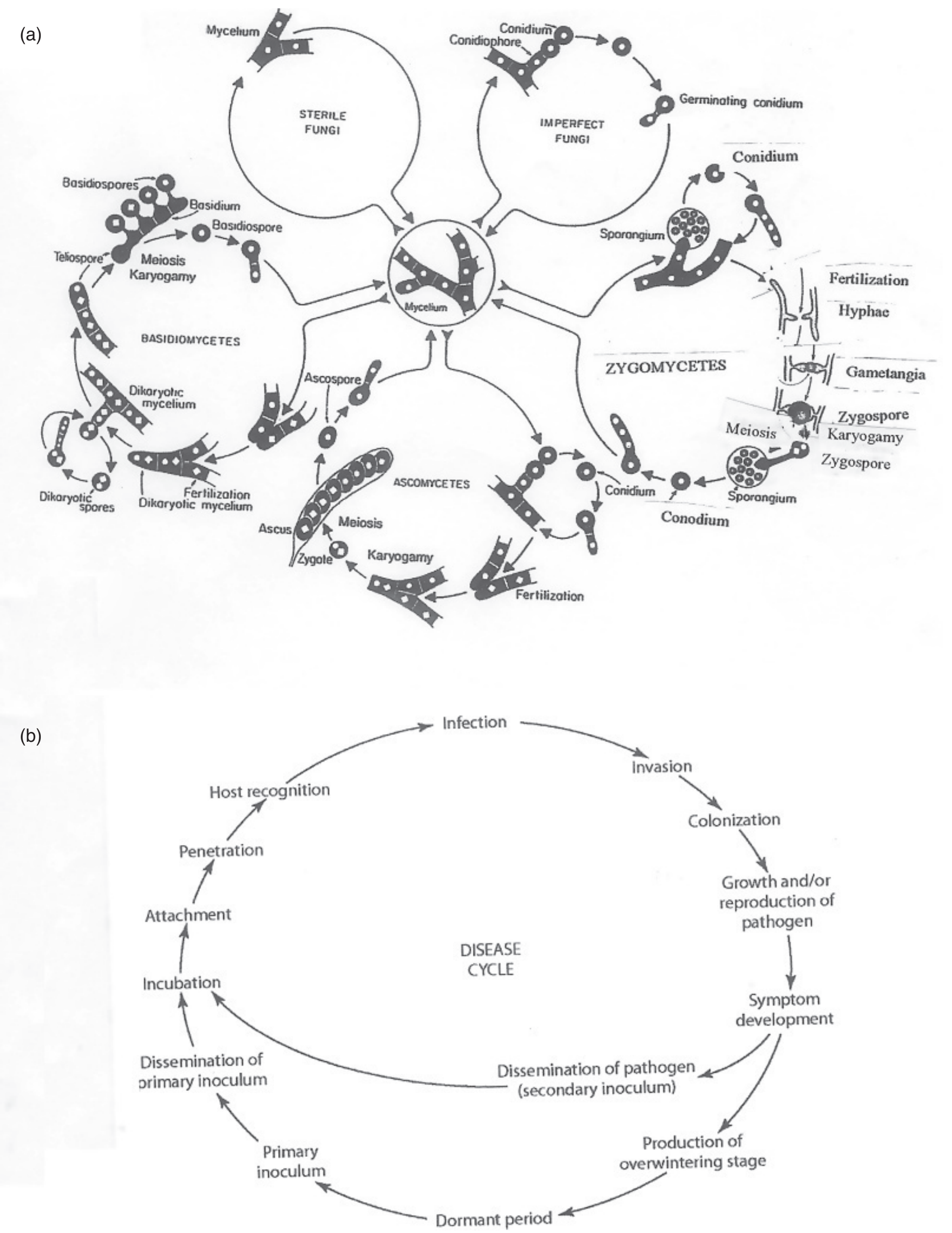
(a)
Mycelium
STERILE FUNGI
Conidium
Conidiophore
Germinating conidium
IMPERFECT FUNGI
Conidium
Sporangium
Fertilization
Hyphae
ZYGOMYCETES
Gametangia
Zygospore
Karyogamy
Meiosis
Zygospore
Sporangium
Conidium
Conodium
Basidiospores
Basidium
Basidiospore
Teliospore
Meiosis
Karyogamy
BASIDIOMYCETES
Dikaryotic mycelium
Dikaryotic spores
Fertilization
Dikaryotic mycelium
Mycelium
Ascospore
ASCOMYCETES
Ascus
Meiosis
Zygote
Karyogamy
Fertilization
(b)
Infection
Invasion
Colonization
Growth and/or reproduction of pathogen
Symptom development
Dissemination of pathogen (secondary inoculum)
Production of overwintering stage
Dormant period
Primary inoculum
Dissemination of primary inoculum
Incubation
Attachment
Penetration
Host recognition
DISEASE CYCLE

Figure 9 (a) Schematic representation of the generalized life cycles of the main groups of plant pathogenic fungi. (b) Stages of development of a disease cycle. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, 8(a) p. 402, 8(b) p. 80.

perithecial fungi, Plectomycetes – cleistothecial fungi, and Loculoascomycetes – ascostromatal fungi. Instead they recommend the use of 10, and possibly 12 taxa in place of the 4 in the previous scheme. Because of the newness of the new terminology and the fact that the literature has so far used the old system, for the purpose of the audience of this volume, we will continue to use the already established scheme, with the exception of a few names, which we will use here. So,

Pezizomycotina – have mycelium that has cross-walls; produce sexual spores (ascospores) within sacs (asci) (e.g., **Figure 11**) that are either naked or contained

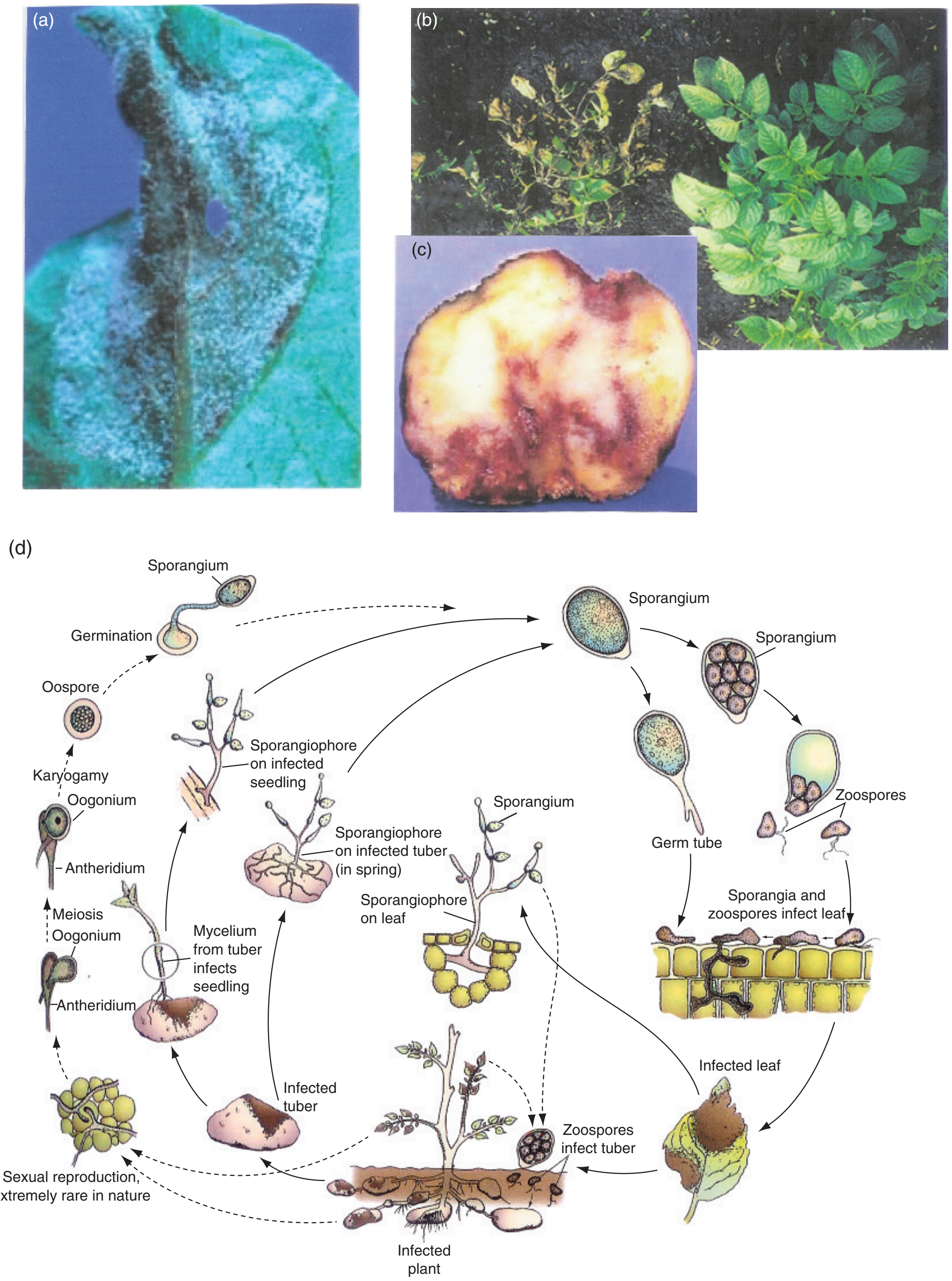
(a)
(b)
(c)
(d)
Sporangium
Germination
Oospore
Karyogamy
Oogonium
Antheridium
Meiosis
Oogonium
Antheridium
Sexual reproduction,
extremely rare in nature
Sporangiophore
on infected
seedling
Mycelium
from tuber
infects
seedling
Infected
tuber
Sporangiophore
on infected tuber
(in spring)
Sporangium
Sporangiophore
on leaf
Sporangium
Sporangium
Germ tube
Zoospores
Sporangia and
zoospores infect leaf
Infected leaf
Zoospores
infect tuber
Infected
plant

Figure 10 Symptoms and the disease cycle of the late blight disease of potato caused by the oomycete *Phytophthora infestans.* (a) Infected potato leaf showing fungal mycelium, sporangia, and sporangiophores. (b) Potato plant resistant to late blight looks almost unaffected (right) compared to a susceptible potato plant which has been killed by the fungus. (c) Rotting of a potato tuber following infection by the late blight. (d) Disease cycle of late blight of potato caused by the oomycete *P. infestans.* Photos: (a) and (d) courtesy of PWeingarten, University of Florida; (b), Cornell University; (c), USDA. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 20, ((b) and (c)) p. 21, (d), p. 425.

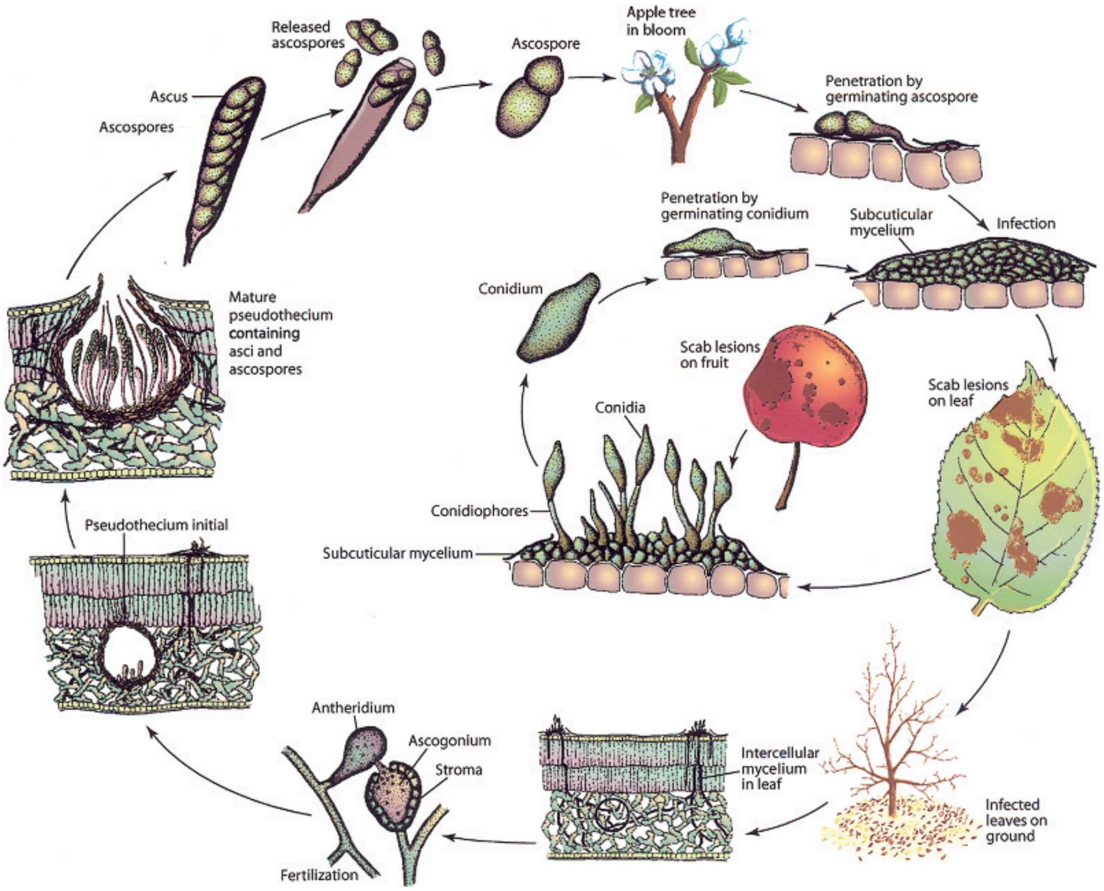
Released ascospores
Apple tree in bloom
Ascospore
Ascus
Ascospores
Penetration by germinating ascospore
Penetration by germinating conidium
Subcuticular mycelium
Infection
Conidium
Mature pseudothecium containing asci and ascospores
Scab lesions on fruit
Scab lesions on leaf
Conidia
Conidiophores
Subcuticular mycelium
Pseudothecium initial
Antheridium
Ascogonium
Stroma
Intercellular mycelium in leaf
Infected leaves on ground
Fertilization

Figure 11 Disease cycle of apple scab disease caused by the ascomycete *Venturia inaequalis.* Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 506. Burlington, MA: Elsevier/Academic Press.

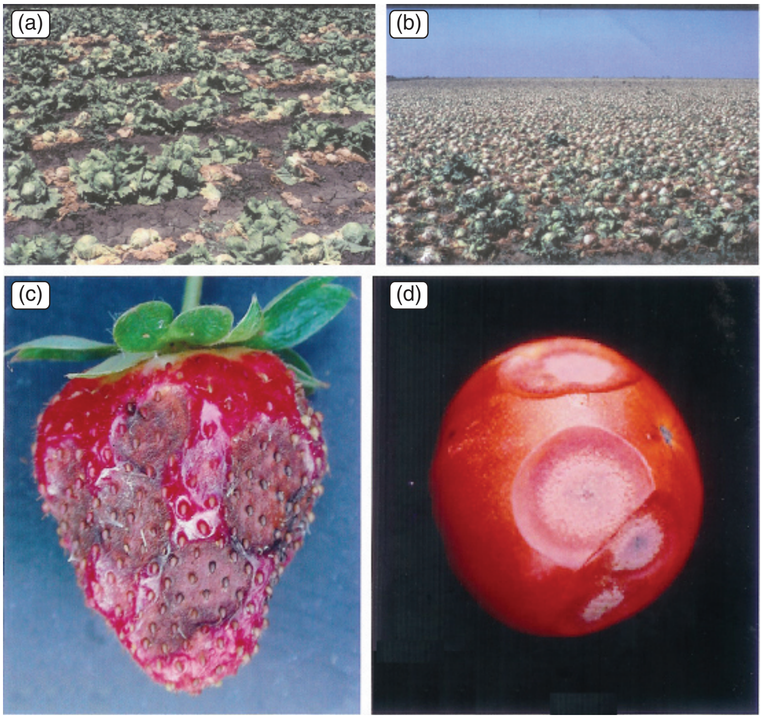

Figure 12 Early (a) and later (b) stages of lettuce infection with the ascomycete *Sclerotinia sclerotiorum* that resulted in total loss of the lettuce crop. (c) Strawberry and (d) tomato fruit showing, at first, lesions and later total rotting following infection of the fruit by the ascomycete *Colletotrichum* as the fruits approach maturity. Photos: (a) and (b) courtesy of KV Subbarao, University of California, Salinas; (c), L Legard; (d), RJ MacGovern, both University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) and (b) p. 271, (c) p. 490, (d) p. 489.

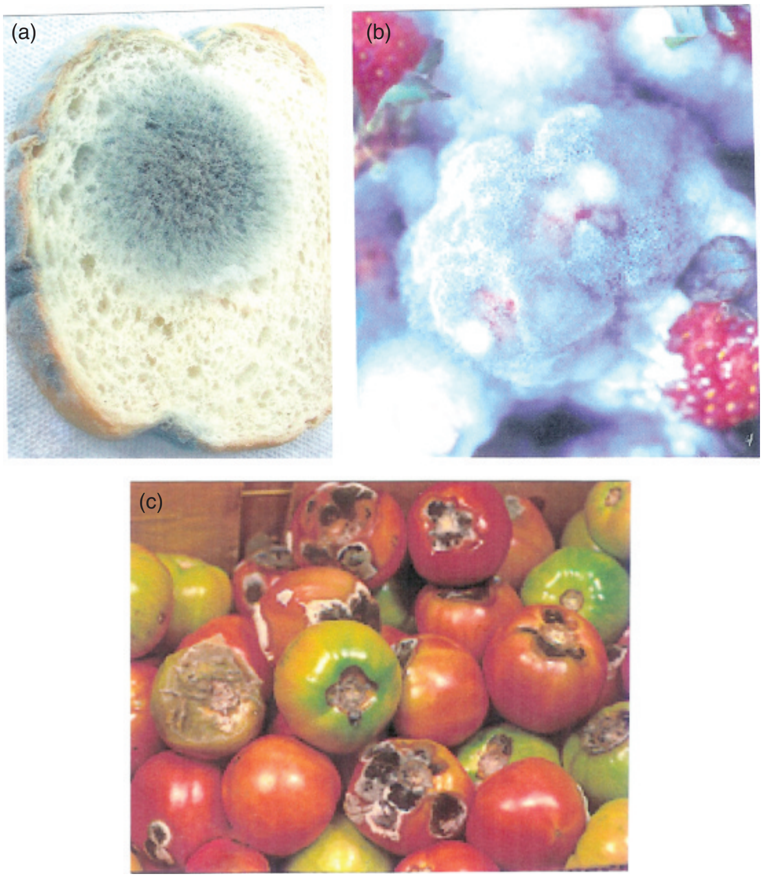

Figure 13 (a) Bread mold caused by the fungus *Penicillium*. (b) Strawberries rotted by the fungus *Rhizopus*. (c) Postharvest rotting of tomatoes by different fungi. Photos: (b) and (c) courtesy of University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 554, (b) p. 13, (c) p. 566.

in fruiting structures of different shapes, namely, cleistothecia, perithecia, and apothecia; produce asexual spores (conidia) on naked hyphae or in containers (pycnidia) or other structures; cause most plant diseases (leaf, stem, and fruit spots and blights, root rots, fruit rots, cankers, vascular wilts, seed rots, etc.).

Important Plant Pathogenic Ascomycetes:

Taphrinales, causing peach leaf curl and plum pockets.

Erisyphales, causing powdery mildews.

Pyrenomycetes, Ascomycetes with perithecia or cleistothecia.

Claviceps, causing ergot (**Figure** 7).

Gibberella (foot rot and stem rot, of corn), Epichloe,

Balansia, Adkinsonella: endophytic on grasses and sedges apple (**Figure** 7(**c**)).

Glomerella (*Colletotrichum* sp.), causing many anthracnose diseases (**Figures** 14(**c**) and 14(**d**)).

Ophiostoma, causing the Dutch elm disease (**Figure** 6).

Cryphonectria, causing chestnut blight (**Figure** 6).

Loculoascomycetes, causing Ascostromata. Asci within locules (cavities).

V. inaequalis, causing apple scab (**Figure** 11).

Discomycetes, causing Ascomycetes with apothecia

Sclerotinia sclerotiorum, causing the white rot or watery soft rot of vegetables (**Figures** 14(**a**) and 14(**b**)).

Athelia (*Sclerotium*) and *Thanatephorus* (*Rizoctonia*), causing root and stem rots of vegetables and fleshy ornamentals and soft rots of fleshy leaves and fruits.

Phylum: **Basidiomycota** – have mycelium, often with binucleate cells, sexual spores (basidiospores) produced externally on a clublike structure called a basidium; some of them produce several types of spores and spore-bearing structures, namely, basidiospores on basidia, spermatia in spermagonia; aeciospores in aecia; uredospores in uredia; and teliospoes in telia; rusts are very serious diseases of grain (**Figures** 12(**a**) and 12(**b**)), of beans and soybeans, and other crops, and of trees. Basidiomycetes also include the smuts of grain crops (**Figures** 12(**a**) and 12(**b**)), and the root rots, wood rots, and decays of trees (**Figures** 12(**c**)–12(**e**)) and timber.

Ustilaginales (the smut fungi);

Ustilago, causing corn smut and loose smut of grains (**Figure** 15(**a**)).

Tilletia, causing covered smut (**Figure** 15(**b**)) or bunt of wheat, and Karnal bunt of wheat.

Uredinales (the rust fungi)

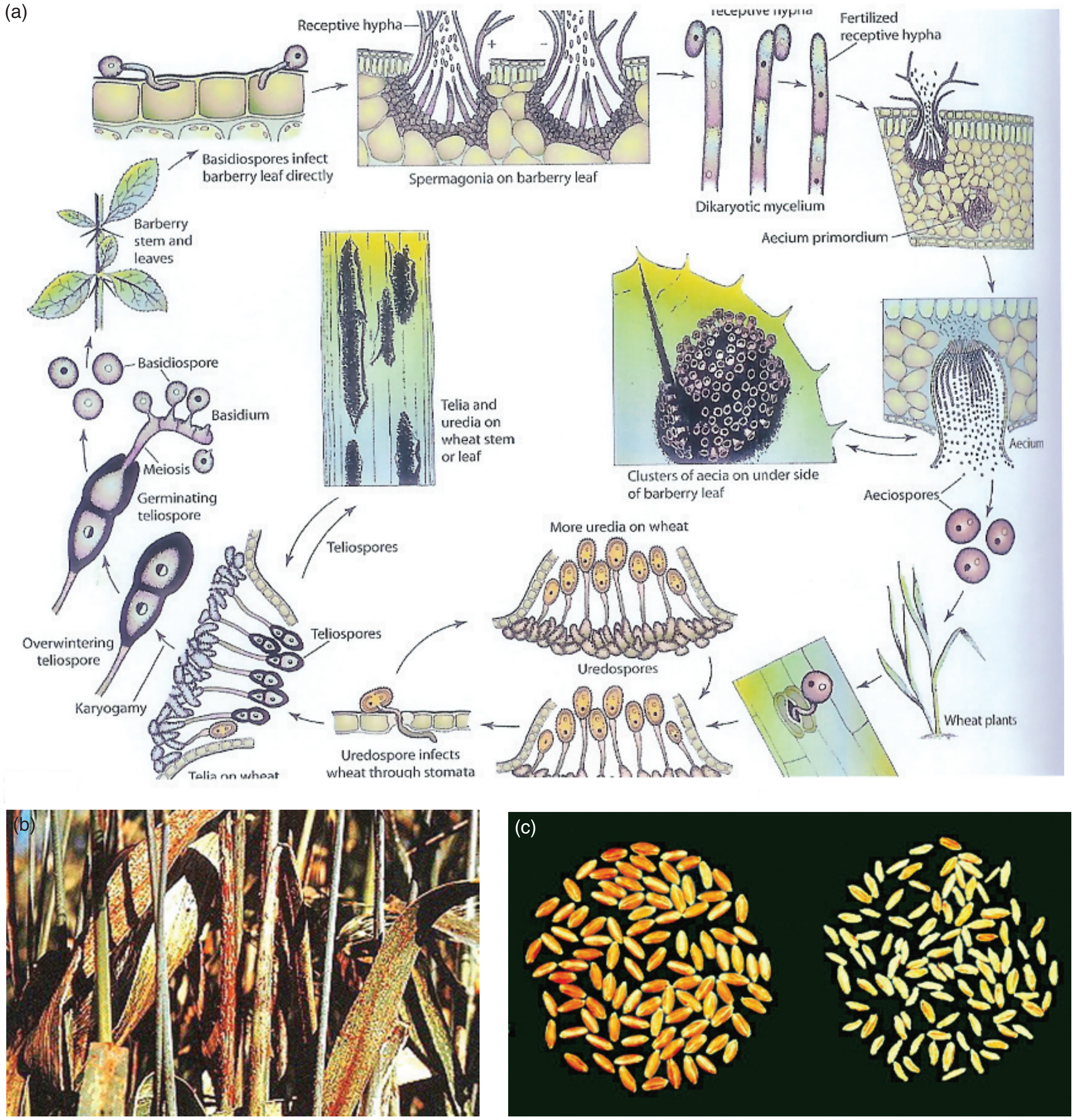
(a)
Receptive hypha
Basidiospores infect barberry leaf directly
Spermagonia on barberry leaf
Fertilized receptive hypha
Dikaryotic mycelium
Aecium primordium
Barberry stem and leaves
Basidiospore
Basidium
Meiosis
Germinating teliospore
Overwintering teliospore
Karyogamy
Telia and uredia on wheat stem or leaf
Teliospores
Teliospores
Telia on wheat
Clusters of aecia on under side of barberry leaf
Aecium
Aeciospores
More uredia on wheat
Uredospores
Uredospore infects wheat through stomata
Wheat plants
(b)
(c)

Figure 14 (a) Disease cycle of stem rust of wheat caused by the basidiomycete *Puccinia graminis.* Notice the variety and sequence of the spores and fruiting bodies, the secondary disease cycle at bottom center, and the need for two alternate hosts, wheat and barberry. (b) Severe infection of wheat by the wheat stem rust fungus, (c) Empty, poor quality kernel from rust-infected wheat plant (left), and wheat kernels from healthy plant. Photos: (b) courtesy of CIMMYT; (c), USDA. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 570, (b) p. 13, (c) p. 566.

Puccinia, causing the devastating rust diseases of cereals, and other plants. *Cronartium*, the rust of pine trees. *Gymnosporangium*, the cedar-apple rust. *Hemileia*, the coffee rust.

Agaricales: The mushrooms; many are mycorrhizal fungi, and many, for example, *Armillaria*, cause losses of about 1 billion dollars in the United States every year.

Ceratobasidiales, causing root rots and decays of trees.

Aphyllophorales, causing wood rots and decays (**Figure 16**).

Prokaryotes: Bacteria and Mollicutes

Approximately 100 species of bacteria and an unknown number of mollicutes cause many severe diseases and losses (**Figures 17** and **18**) in plants. Bacteria and mollicutes are prokaryotic organisms, that is, they do not have organized nuclei bound by a membrane and their DNA is organized as a single chromosome present in an area of the cytoplasm called nucleoid. They also have circular

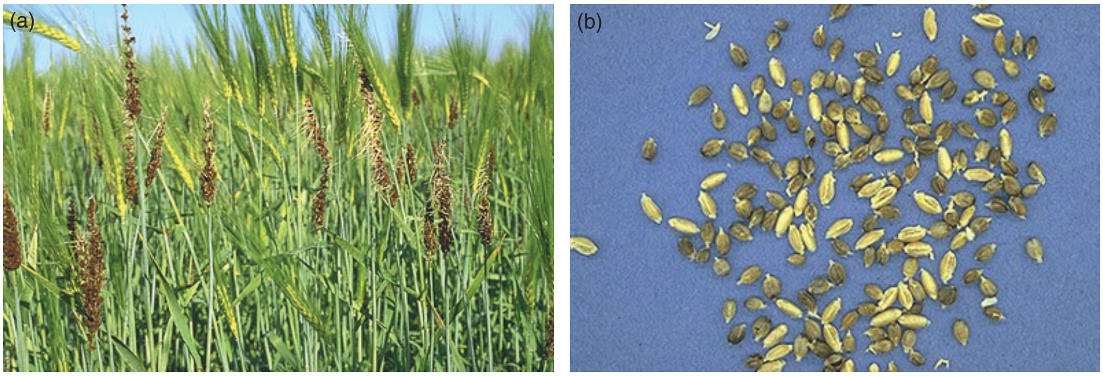

Figure 15 (a). Field symptoms of barley heads infected with loose smut fungus *Ustilago*. (b) Kernels of wheat infected with and carrying teliospores of the cover smut fungus *Tilletia* compared with a few healthy whitish kernels. Photos: (a) courtesy of P Thomas; (b), PE Lipps, Ohio State University. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) and (b) p. 12.

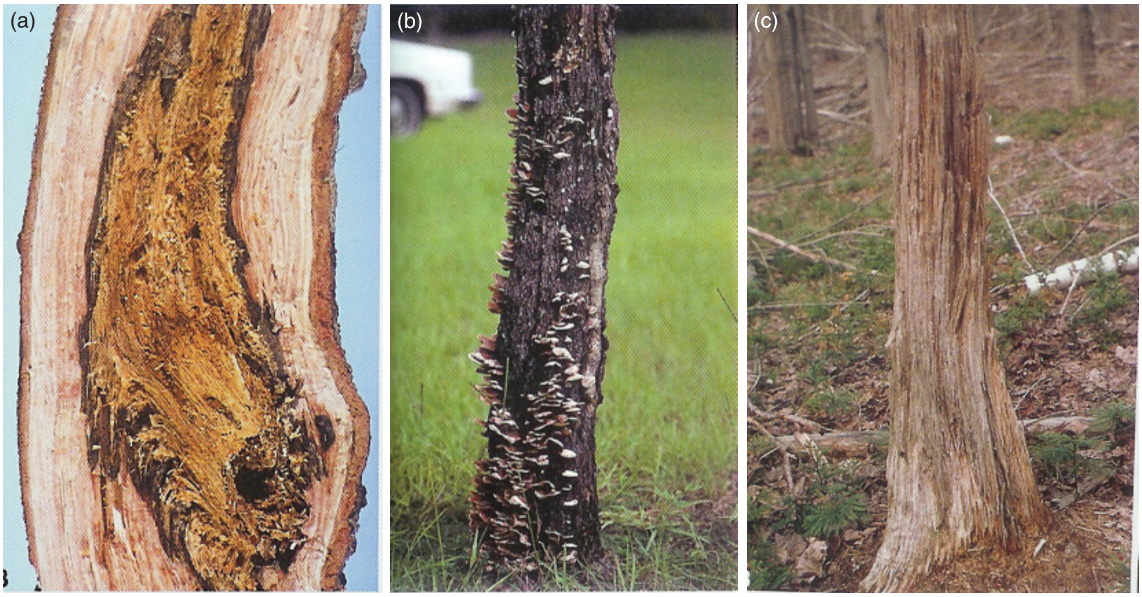

Figure 16 Three stages or types of rotting and decay of trees by wood rotting fungi. Photos: (a) and (c) courtesy of EL Barnard, Florida Department of Agriculture and Forestry; (b), University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 608, (b) p. 607, (c) p. 609.

pieces of DNA contained in plasmids and mitochondria. Most plant pathogenic bacteria are necrotrophs, that is, they are facultative saprophytes, and can be grown on synthetic nutrient media, but survive and multiply best in contact with their host plants. Some fastidious vascular bacteria, however, survive in nature only inside their host plants, in either the xylem vessels or the phloem sieve tubes.

It is estimated that the plant pathogenic bacteria comprise between 30 and 100 species. Most plant pathogenic bacteria are facultative parasites or necrotrophs and are easy to grow on artificial media. Several, known as fastidious bacteria, are difficult to grow on common nutrient media but can be cultured on specialized complex media. Most bacteria attack tissues at or near the surface of a host plant but the fastidious bacteria grow in the xylem vessels of the plant. The mollicute phytoplasmas and spiroplasmas, as well as some rather fastidious bacteria, grow only in the phloem of the plant.

Plant pathogenic mollicutes also survive and multiply in nature only inside the phloem sieve tubes of their living hosts (**Figure 19**). Mollicutes of only one genus, *Spiroplasma*, can be grown in culture; all others, usually referred to as phytoplasmas, can be maintained but do not multiply on nutrient media.

One rather common plant pathogenic bacterium, *Agrobacterium tumefaciens*, which causes the crown gall disease (**Figure 4(c)**) in many plants but can survive freely in

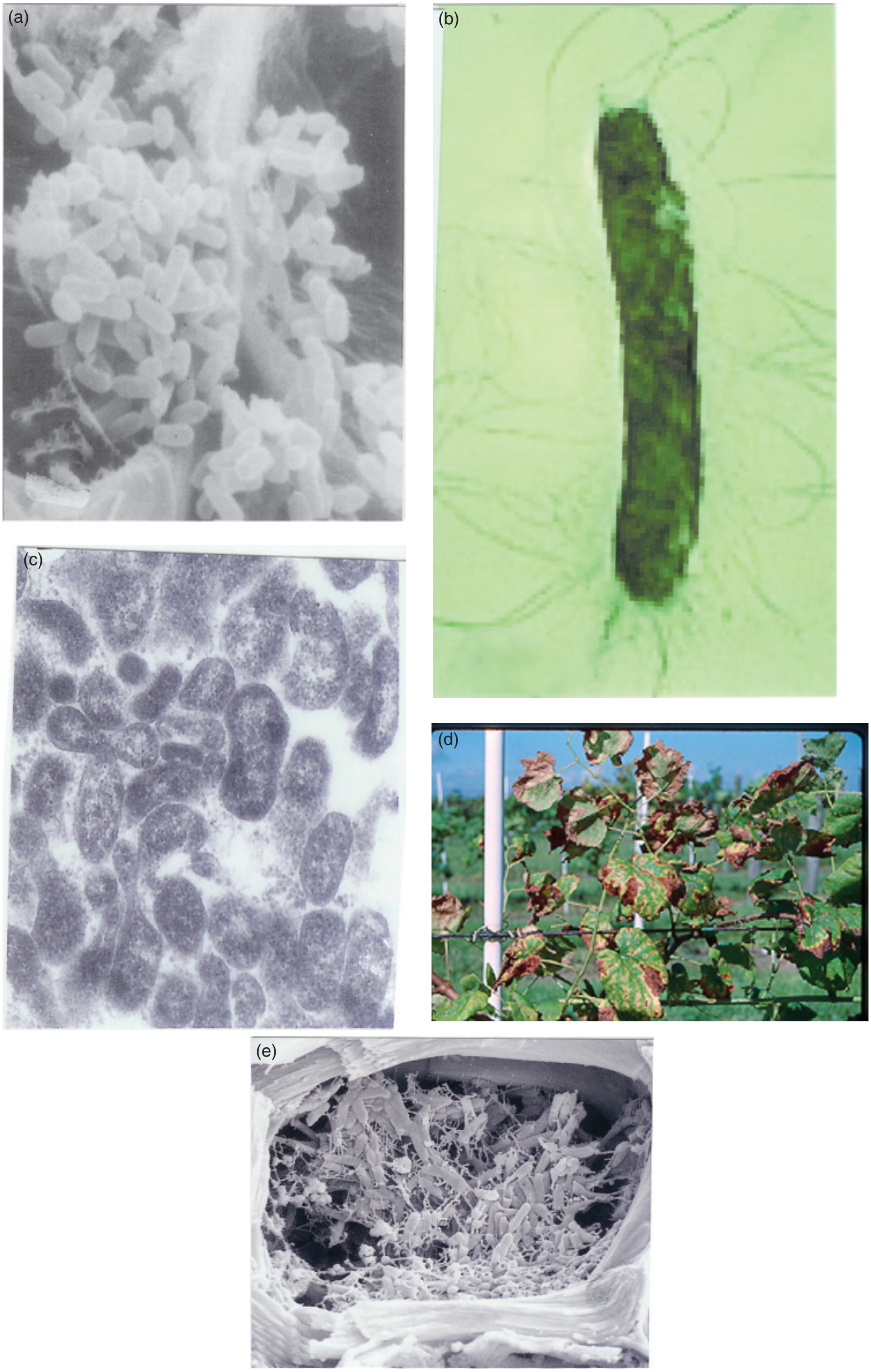

Figure 17 (a) Typical rod-shaped plant pathogenic bacteria. (b) Bacterium with peritrichous flagella. (c) Mollicutes in a phloem vessel. (d) A grapevine with scorched leaves caused by the xylem-limited bacterium *Xylella*. Photos: (a) courtesy of Roos and Hatting, The Netherlands; (b), Oregon State University; (c), JE Worley, USDA; (d), DL Hopkins, University of Florida; (e), E Alves, Federal University, Lavras, Brazil. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (b) p. 645, (c) p. 9, (d) p. 680, (e) p. 681.

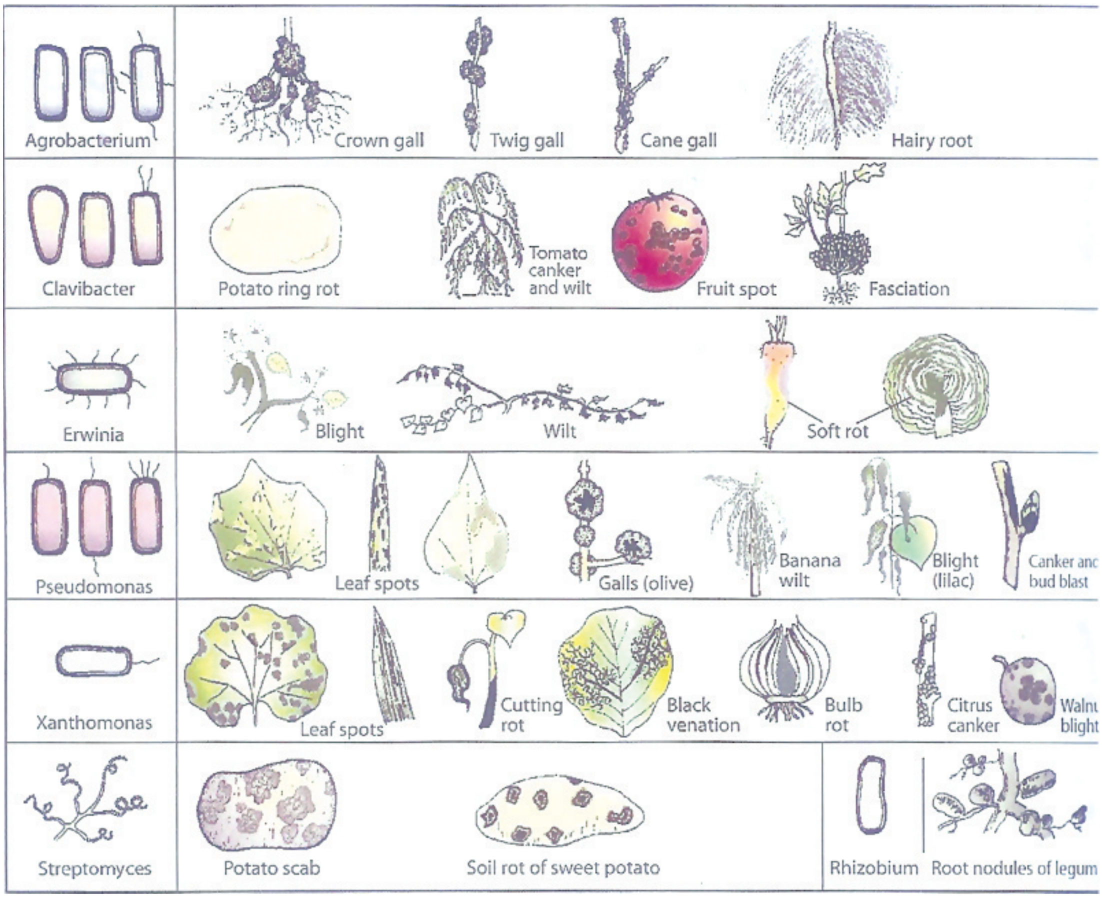
Agrobacterium
Crown gall
Twig gall
Cane gall
Hairy root
Clavibacter
Potato ring rot
Tomato canker and wilt
Fruit spot
Fasciation
Erwinia
Blight
Wilt
Soft rot
Pseudomonas
Leaf spots
Galls (olive)
Banana wilt
Blight (lilac)
Canker anc bud blast
Xanthomonas
Leaf spots
Cutting rot
Black venation
Bulb rot
Citrus canker
Walnu blight
Streptomyces
Potato scab
Soil rot of sweet potato
Rhizobium
Root nodules of legum

Figure 18 (a) Relative morphology of the most important genera of plant pathogenic bacteria and the symptoms they cause on their host plants. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 620. Burlington, MA: Elsevier/Academic Press.

the soil for more than a year after infection, has been proven to be a natural genetic engineer and the bacterium and its purified DNA have been used to multiply and to transfer DNA from some plants and other organisms into particular plants. This was and still is one of the most important steps and contributions of plant pathology to the huge development of genetic engineering of plants and of plant biotechnology.

Morphology

Most plant pathogenic bacteria are rod-shaped, considerably smaller than fungi (**Figures 4**, **17**, and **19**), ranging from 0.5–1.0 μm in diameter to 0.6–3.5 μm in length. In some bacteria, and in older cultures, the bacteria may be longer and they may be branched. Bacteria of the genus *Streptomyces* are filamentous. Most species of bacteria have one or more flagella that can be found at the polar ends or cover the entire surface of the bacteria (**Figure 19**(**b**)).

Although bacterial cells are contained by a cell wall, which gives them the typical rod shape, plant pathogenic mollicutes lack a cell wall and thereby take on a shape that is spherical, tubular, or polymorphic (**Figures 3**, **4**, and **17**).

Reproduction

Plant pathogenic bacteria and mollicutes reproduce asexually by fission. That is, following the replication of the DNA in the bacterium, the cell wall of the bacterium also divides, separating the two halves of the bacterium into two new identical bacteria (**Figure 2**). Any plasmids present in the bacterium replicate independent of the chromosomal DNA, but may code for proteins that play an important role in the development of disease. Although bacteria do not have a typical sexual reproduction, genetic change in their DNA is introduced by conjugation of two identical or different bacteria, during which segments of the DNA from one bacterium are transferred to the other bacterium; by bacteria-infecting viruses (bacteriophage); and most commonly by mutation, that is, mistakes that occur during replication of the DNA.

Classification of plant pathogenic bacteria and mollicutes

Because most bacteria lack distinctive morphological characteristics, their taxonomy and names are less stable than in other organisms. Scientists, however, have developed an array of diagnostic techniques for

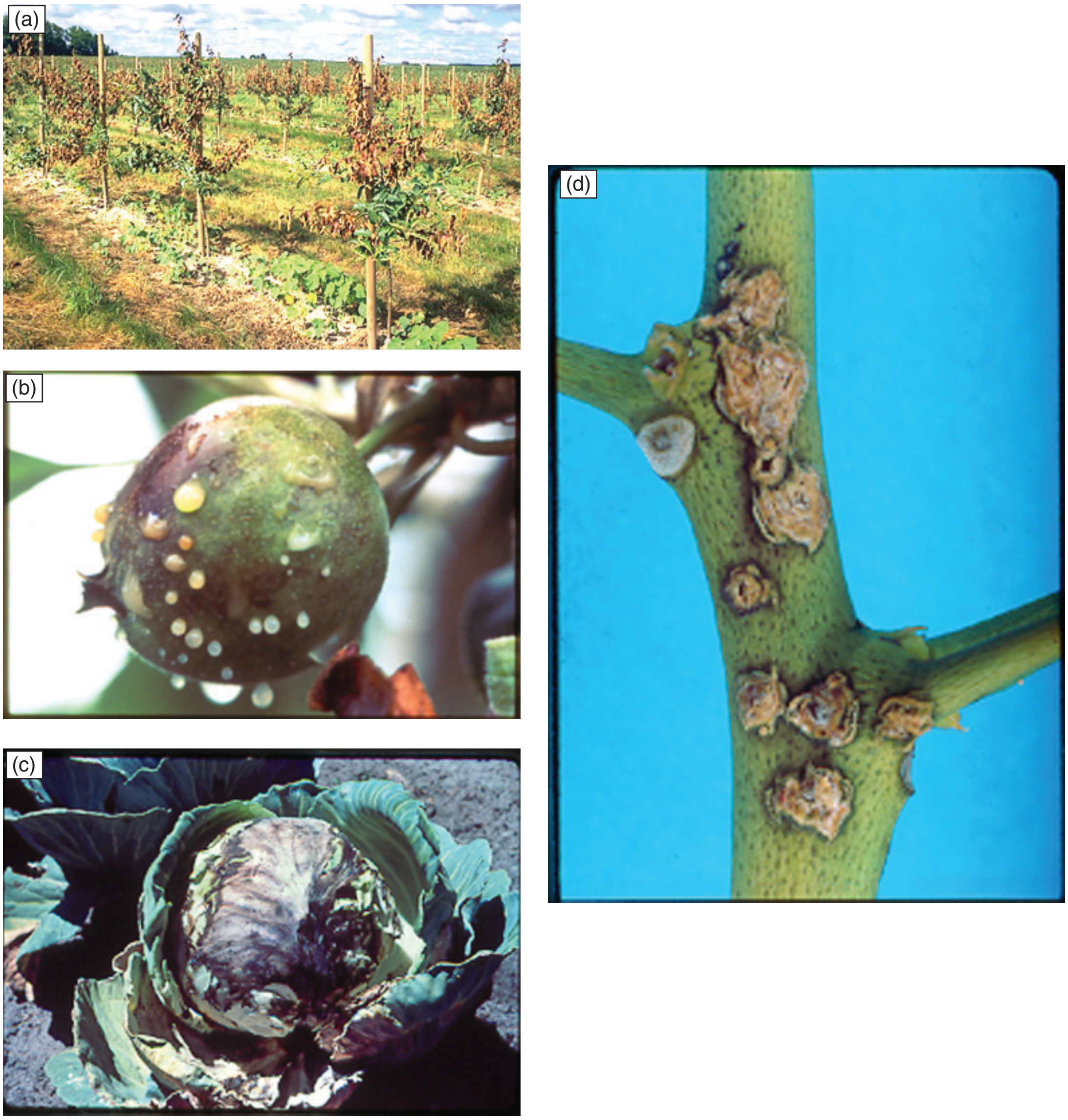

Figure 19 (a) A young apple orchard destroyed by fire blight caused by the bacterium *Erwinia amylovora*. (b) Infected apple fruit exuding droplets of fire blight bacteria. (c). Bacterial soft rot of vegetables, for example, cabbage, caused by at least three species of bacteria. (d) Symptoms of citrus canker on a young stem. Photos: (a) and (b) courtesy of T Van Der Zwet, USDA; (c), Department of Plant Pathology, University of Florida; (d), Division of Plant Industry, Florida Department of Agriculture. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 644, (b) p. 645, (c) p. 658, (d) p. 673.

bacteria, including serological and molecular techniques that are very effective in bacterial taxonomy and classification.

The most common prokaryotic pathogens of plants can be classified approximately as shown below. The shapes of the bacteria and the kinds of plant symptoms caused by most important bacteria are shown in **Figures 5** and **18**.

Kingdom: Prokaryotae

Bacteria: Have a cell membrane and a cell wall.

Gram-negative bacteria

Family: Enterobacteriaceae. Selected important genera.

Erwinia – causing fire blight of apples and pears, nursery stock, vascular wilts, and soft rots of fleshy fruits, vegetables, and ornamentals (**Figure 17**(**b**)).

Pseudomonas – causing numerous leaf spots, blights, wilts, and so on. (**Figure 17**(**a**)).

Ralstonia – causes wilt of solanaceous crops.

Xanthomonas – causes leaf spots, blights, and citrus canker.

Family: Rhizobiaceae

Genus: *Agrobacterium, A. tumefaciens* (**Figure 5**(**c**)) causing the crown gall disease.

Family: Still unknown;

Genus: *Xylella fastidiosa*. Xylem - inhabiting, causing leaf scorch and dieback in trees (**Figures 17**(c)–**17**(e).

Gram-positive bacteria

Genus: *Clavibacter* – causing bacterial wilts in alfalfa, potato, and tomato.

Genus: *Streptomyces* sp. – causing the common scab of potato. Antibiotics.

Mollicutes: Have cell membrane but not cell wall

Family: Still unknown

Genus: *Phytoplasma* (**Figure 17**(**c**)), causes numerous yellows, proliferation, and decline diseases in trees and some annuals.

Family: Spiroplasmataceae

Genus: *Spiroplasma*, causes corn stunt, citrus stubborn disease.

Viruses and Viroids

More than 2000 viruses causing disease in plants have been identified and their properties have been studied. Viruses are the smallest and simplest pathogens. Although viroids are much smaller and simpler than even viruses (**Figures 20** and **21**), the ability of viroids and viruses to cause disease and losses from disease is second only to those of fungal pathogens. Plant viruses cause a wide variety of symptoms on the plants they infect. When plants infected with the virus are more or less resistant to the virus, they may limit the spread of the virus and produce numerous small local lesions (**Figure 21**(**a**)), which, usually, but not always, keep the virus limited to the lesions and do not allow it to spread systemically through the plant and cause systemic symptoms. When the plant is susceptible to the virus and becomes infected, the virus may spread internally through parts of, or more commonly throughout, the plant and the plant then develops symptoms appearing as leaf mosaic (**Figures 21**(**c**) and **21**(**d**)), as necrotic leaves and poor growth of the plant, as internal necrotic veins (**Figure 21**(**i**)), as yellowing of leaves and severe stunting of the plant (**Figures 21**(**f**) and **21**(**h**)), as ringlike or amorphous, discolored, bumpy or necrotic patches on fruit and seeds (**Figure 21**(**g**)), and many others. In some cases, viruses also produce internal symptoms in the epidermal cells of the leaves of their hosts, appearing as amorphous or crystalline inclusions (**Figure 21**(**b**)) in the cytoplasm or the nucleus of the plant cells. The inclusions are characteristic of the genus of the virus and in many cases are used in the detection and identification of the virus.

Although plant viruses are not known to infect humans and animals – with the exception of some of their insect vectors – the study of plant viruses, and particularly the tobacco mosaic virus (TMV) (**Figures 20**(**a**) and **22**(**a**)), causing the tobacco mosaic disease, has contributed immensely to our knowledge and understanding in several areas of biology, genetics, plant pathology, plant and animal science,

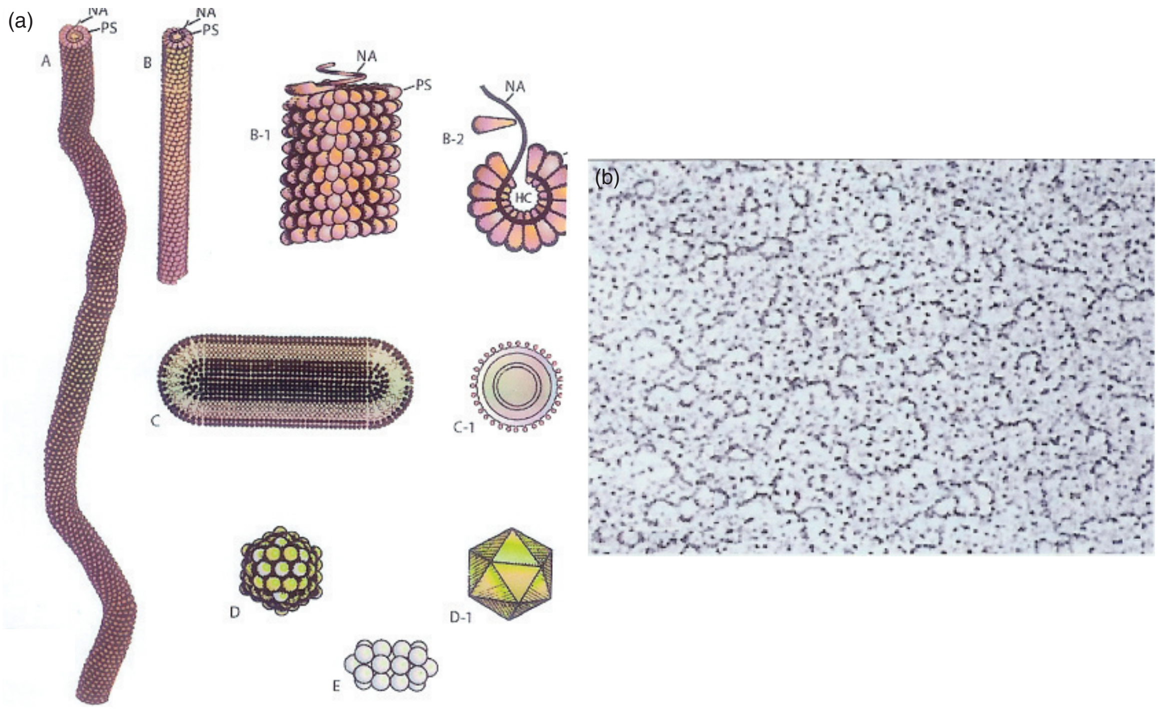

Figure 20 (a) Diagram of shapes and relative sizes of plant viruses. AA&C, flexuous, threadlike virus; NA, nucleic acid; PS, protein subunit. (a) Rigid rod-shaped virus: B-2 = cross-section of the virus showing the arrangement of the nucleic acid. (b) Viroids (small circles and equivalent length lines). The viroid that causes the coconut cadang-cadang (dying, dying). Photo (b) courtesy of JW Randles. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 729, (b) p. 822.

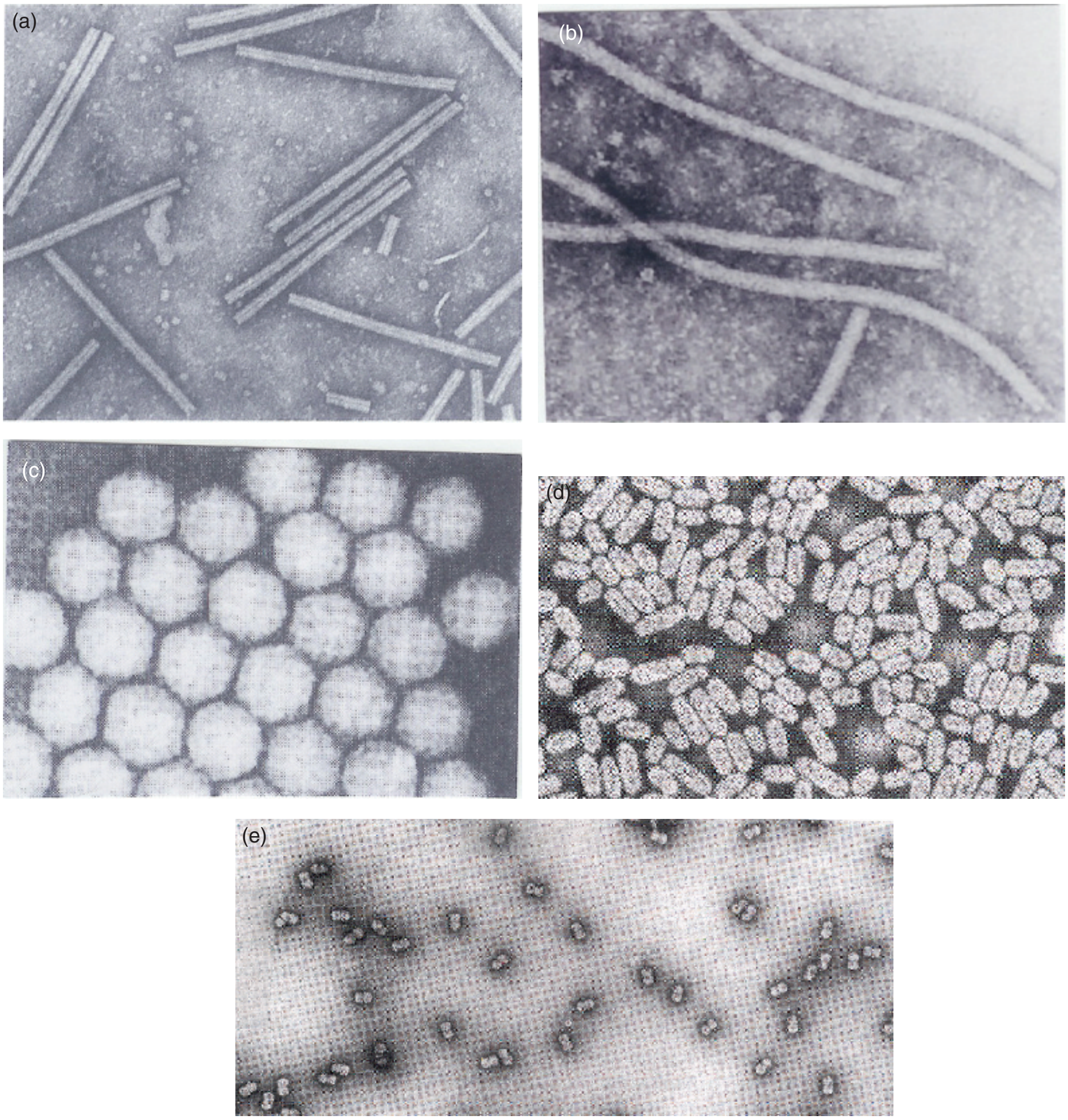

Figure 21 The shapes and relative sizes of plant viruses. (a) Rod-shaped virus, (b) filamentous flexuous virus, (c) isometric polyhedral virus, (d) short, bacillus-like virus, and (e) geminivirus. Photos: (d) courtesy of EMJ Jaspars, The Netherlands; (f), E Hiebert, Department of Plant Pathology, University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 759, (c) p. 793, (d) p. 728, (e) p. 806.

medicine, biochemistry, and others. The reason for these successes of TMV is that this virus was the first to be detected and was hardy enough to withstand rough experimental treatment. At first it appeared to investigators to be an infectious fluid, then to be or contain elongated particles composed of infectious protein. It was soon shown to contain not only protein but also nucleic acid (RNA). It was subsequently shown that infectivity resided in the nucleic acid only while the protein played primarily a protective role for the nucleic acid. Subsequently, it was studies of TMV that showed that the virus could be measured through counting the local lesions produced by a virus preparation and that the virus consisted of cylindrical rod-shaped particles visible under the electron microscope. Soon after, biochemical and genetic studies of TMV showed that the protein subunits of the virus coat were attached to the viral RNA and that the size and other properties of each protein were determined by the sequence of the triplets of the four nucleotides along a stretch of the viral RNA containing three times as many nucleotides as the number of amino acids in the protein subunits. This suggested that each amino acid of the protein subunit corresponded to three nucleotides of the RNA. The study of the sequence of nucleotide triplets on the RNA to the slight changes of amino acids in the coat protein subunits of the various viral strains showed that each change in one or more of the three nucleotides corresponded to always the same changes in amino acids of the protein subunits. This information, gained from the study of TMV, led to the discovery of the genetic code in viruses, which was later shown to be the same in all organisms.

Characteristics of plant viruses and viroids

Viruses consist of one or a few molecules of nucleic acid (RNA or DNA but never of both being present in the same virus), whereas viroids have only a small naked RNA (**Figure 20**(**b**)). Plants are also affected by about 40–50 viroids that have only an RNA that is about 10 times smaller than that of viruses, is free or naked, and not contained in a protein coat. Both, viruses and viroids can infect plants, replicate themselves, and cause disease. Both viruses and viroids are intracellular parasites. Only a few of them survive in plant debris or outside plant cells but even they do not multiply there. Viruses and viroids are too small to be seen even with the compound microscope. Therefore, TMV was the first virus to be detected and identified by several indirect techniques, which include kind of symptoms on infected plants, presence of characteristic inclusion bodies in infected young tissues, transmission of symptoms to healthy plants by sap, grafting, specific insect and other vectors, and using serological tests or nucleic acid probe tests against known viruses. In sap from infected cells and in purified preparations, most viruses and viroids can be seen under the electron microscope.

Morphology

Nearly half of the known viruses are rigid rodlike, 15 × 300 nm (nanometers) (**Figure 20**(**a**)), or flexuous

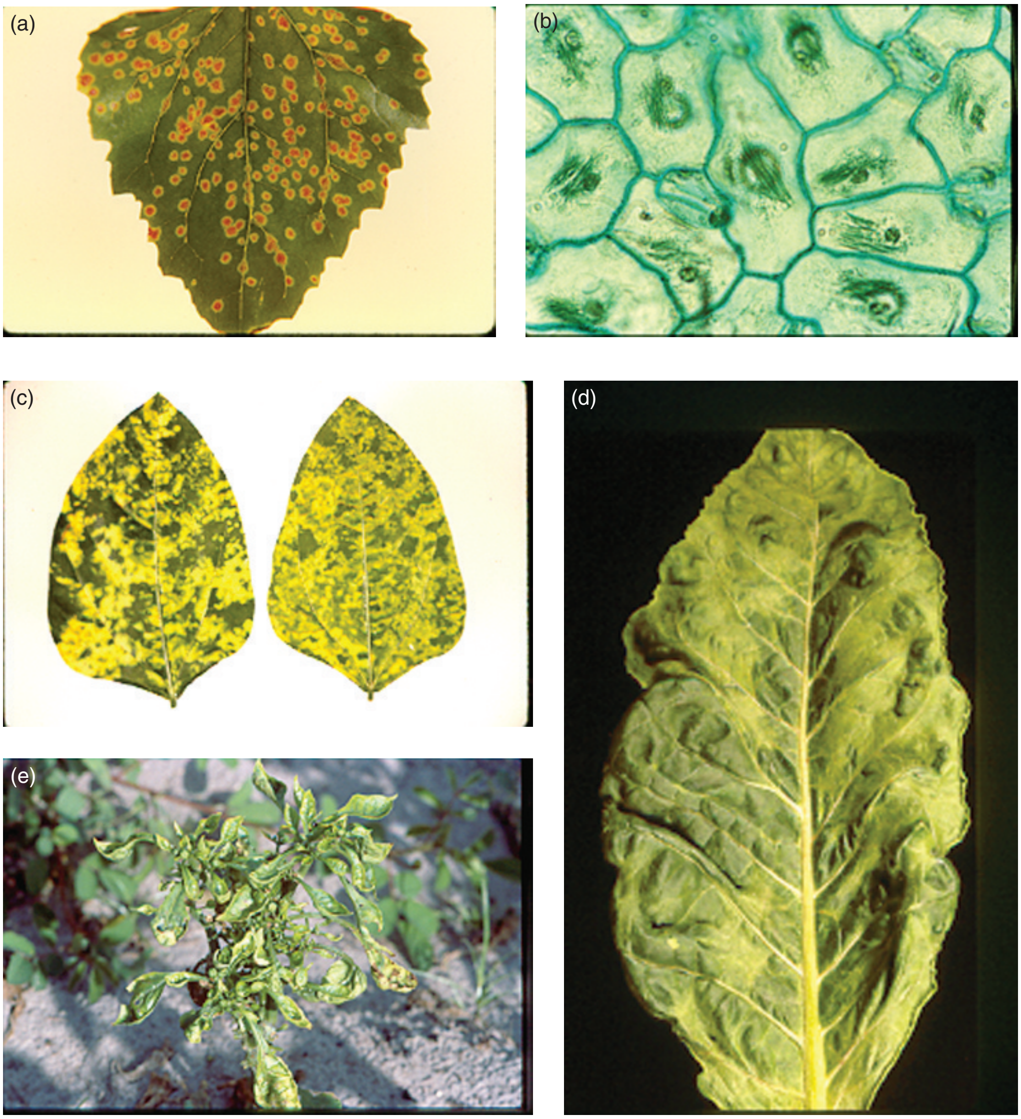

Figure 22 (*Continued*)

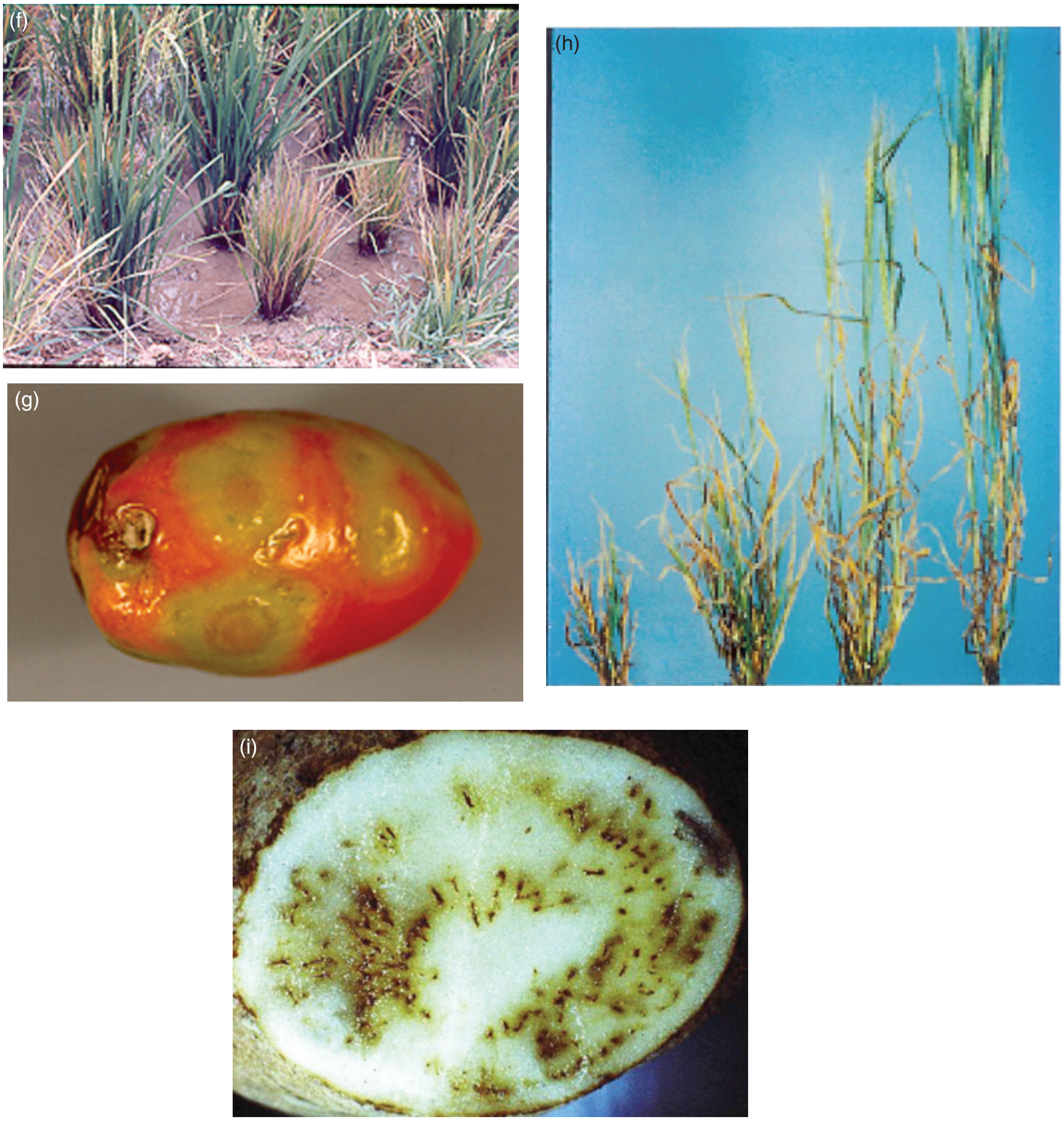

Figure 22 (a) Local lesions on leaf of *Chenopodium* (lamb's quarters) plant hand-inoculated with sap from *Potato virus Y*-infected plant. (b) Cellular inclusions produced by cells of some plant inoculated with certain viruses. (c) Cowpea leaf showing typical mosaic symptoms. (d) Mosaic on tobacco mosaic virus-infected tobacco. (e) Mosaic and foliar malformation on pepper leaves infected with *Potato virus Y*. (f) Rice plants infected with the grassy rice stunt virus and showing severe stunting, yellowing, and excessive tillering. (g) Tomato fruit showing ringlike and other malformations caused by the Tomato spotted wilt virus. (h) Barley yellow dwarf symptoms of varying severity on barley plants. (i) Potato tuber showing vein necrosis caused by the potato leaf roll virus. Photos: (b) courtesy of R. Christi; (d), (e), and (g) courtesy of Department of Plant Pathology, University of Florida; (f), H Hibino; (h), WF Rochow, Cornell University; (i), Department of Plant Pathology, University of Idaho. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 736, (b) p. 726, (c) p. 725, (d) p. 759, (e) p. 765, (f) p. 800, (g) p. 796, (h) p. 782, (i) p. 783.

threads about 11 nm in diameter by lengths varying from 480 to 2000 nm (**Figure 20(b)**). Almost as many viruses are spherical, actually isometric or polyhedral, about 17–100 nm in diameter (**Figure 20(c)**), and a few are bacillus-like rods 52 × 75 nm in diameter and 300–380 nm long (**Figure 20(d)**) and some are monopartite or bipartite geminiviruses (**Figure 20(f)**). Many viruses have split genomes, that is, they consist of two or more distinct nucleic acid strands, each encapsidated in similar or different sized particles made of the same protein molecules (protein subunits). The surface of viruses consists of a definite number of protein subunits, which are spirally arranged in the elongated viruses and packed on the sides of polyhedral viruses. In cross-sections, the elongated viruses appear as hollow tubes of spirally arranged protein subunits, with the nucleic acid strand embedded between successive spirals of protein subunits. In spherical viruses, the visible shell consists of protein subunits, whereas the nucleic acid is inside the shell but it is not known how it is arranged. Viruses are not cells and have neither the cytoplasm nor the nucleus, but the rhabdoviruses and a few spherical viruses have an outer membrane composed of lipoprotein.

Reproduction (replication)

Plant viruses and viroids replicate only within living plant cells or protoplasts. The mechanisms of replication are quite complex and the complexity varies depending on whether the nucleic acid of the virus is RNA or DNA, single- or double-stranded, and positive or negative strand. Basically, in the replication of viruses and viroids, the infected plant cell provides all the structural materials (nucleic acid and proteins), the energy, and the machinery needed to make more viruses, whereas the virus or viroids supply only the blueprint in the form of their nucleic acid. Virus (and viroid) replication is analogous to making copies of an original page (the virus) with the help of a modern copier (the plant cell). Once new viral nucleic acid and viral protein are produced in the cell, they are assembled into virus particles (**Figure 20**). The virus particles, in turn, either accumulate in the cell singly or as parts of inclusion bodies, or they move to other adjacent plant cells and, possibly through the phloem sieve elements, throughout the plant.

Classification

The main characteristics considered in the classification of viruses are the nature and number of their nucleic acids and the shape and size of their particle(s). A very sketchy classification of plant viruses is as follows:

Kingdom: Viruses

RNA Viruses

Single-stranded positive RNA

Rod-shaped particles – for example, *Tobamovirus* (TMV).

Filamentous particles – for example, *Potyvirus* (potato virus Y).

Isometric particles – for example, *Luteovirus* (barley yellow dwarf virus).

Single-stranded negative RNA – for example, potato yellow dwarf virus.

Buniaviridae – for example, *Tospovirus* (tomato spotted wilt virus).

Double-stranded RNA

Isometric viruses – for example, *Reoviridae*, Phytoreovirus (wound tumor virus)

DNA Viruses

Double-stranded DNA

Isometric – for example, *Caulimovirus* (Cauliflower mosaic virus).

Nonenveloped bacilliform – for example, *Badnavirus* (rice tungro bacilliform virus).

Single-stranded DNA

Geminate (twin) particles – for example, *Geminivirus* (bean golden mosaic virus).

Single isometric particles – for example, *Nanovirus* (banana bunchy top virus)

Taxonomy (Grouping) of Viroids. (**Figure 21**(**b**)) – small, single-stranded, circular RNAs that replicate and cause disease in plants. There are no known viroids infecting humans or animals.

Parasitic Higher Plants

More than 2500 species of higher plants live parasitically on other higher plants. Relatively few of these plants, however, affect and cause disease on cultivated higher plants or on forest trees of commercial significance. The parasitic plants produce flowers and seeds like all plants. They belong to widely separated botanical families and vary greatly in dependence on their host plants. Some parasitic plants (e.g., mistletoes) have chlorophyll but no roots; therefore, they depend on their hosts only for water and inorganic nutrients. Others (e.g., dodder) have little or no chlorophyll and no true roots; therefore, in addition to water and inorganic nutrients, they also depend on their hosts for photosynthetic products. Parasitic higher plants obtain water and nutrients from their hosts by producing and sinking into the vascular system of their host stems or roots food-absorbing organs called haustoria. The following are the most important parasitic higher plants and the botanical families to which they belong.

Cuscutaceae – *Cuscuta* sp., the dodders (**Figure 23**)

Viscaceae – *Arceuthobium*, the dwarf mistletoes of the conifers

Phoradendron, – the American true mistletoe of broad-leaved trees

Viscum – the European true mistletoes

Orobanchaceae – *Orobanche*, the broomrapes of tobacco

Scrophulariaceae – *Striga*, the witchweeds of many monocotyledonous plants.

Parasitic higher plants vary in size from a few millimeters to a few centimeters in diameter and from 1 cm tall to upright green plants more than 1 cm tall. Some, however, are yellow or orange leafless vine strands that may grow to several meters in length and entwine around the stems of many adjacent host plants. Parasitic higher plants reproduce by seeds. Seeds are disseminated to where host plants grow by wind, runoff water, birds, and cultivating equipment. Seeds of some parasitic plants are forcibly expelled to significant distances (10 m or more). Parasitic higher plants overwinter on perennial hosts or as seeds on the hosts or on the ground. In spring, the seeds germinate and the seedling infects a new host plant. Control or management of parasitic higher plants depends on removing infected plants carrying the parasites and avoiding bringing seeds of parasitic plants into new areas.

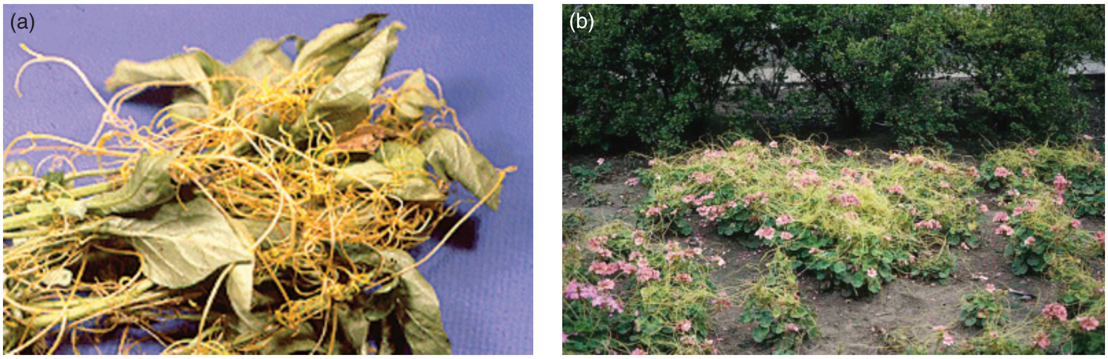

Figure 23 (a) Parasitic higher plant, dodder, of the species *Cuscuta*, has entwined itself around a pepper plant, which has been overcome. (b) A small field of geraniums attacked by dodder. Photo courtesy of Department of Plant Pathology, University of Florida. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 707.

Plants growing in the wild often suffer damage caused by other plants growing over the first plants and covering them so completely that they cannot get any sunlight. As a result such plants decline and die prematurely (**Figure 23**).

Parasitic Green Algae

Algae are the organisms, often microorganisms, other than typical land plants, that can carry on photosynthesis. Algae are sometimes considered as protists with chloroplasts. Some algae, the so-called blue-green cyanobacteria, belong to the kingdom Prokaryotes but most of them, that is, the rest, belong to the kingdom Chromista. Algae are the main producers of photosynthetic materials in aquatic ecosystems, including unstable systems such as muds, sands, and intertidal aquatic habitats. Green algae are single-celled organisms that form colonies, or multicellular free-living organisms, all of which have chlorophyll b.

Several algae are pathogenic of other organisms. For example, cyanobacteria cause the black band disease that leads to the bleaching and death of coral symbionts of the algae. Many red algae are parasites on other, mostly related red algae. Colorless green algae of the genus *Prototheca* cause skin infections in humans. Most of the green algae live as endophytes of many hydrophytes to which they seem to cause little or no damage. A few genera of green algae are parasitic on higher plans.

Nematodes

Nematodes are usually microscopic animals that are wormlike in appearance but quite different taxonomically from true worms. Most nematode species live freely in fresh- or saltwater but numerous species attack and cause disease on humans and animals and several hundred species feed primarily on roots of plants and cause disease in them. Symptoms of infected plants include the following (**Figure 24**): root lesions (**Figure 24**(**a**)) or root galls (**Figure 24**(**c**)); devitalized root tips; excessive root stunting or branching; distortion of above-ground plant organs and death of the plant (**Figure 24**(**d**)); and, in some cases (**Figures 25**(**a**) and **25**(**b**)), death of branches and whole trees. The annual worldwide losses of crops caused by nematodes on various crops are more than 80 billion dollars.

Morphology

Plant parasitic nematodes are small and eel-shaped but only 300–1000 μm long, with some up to 4 mm long × 15–35 μm wide (**Figure 26**). The females of some of these nematodes become swollen at maturity and have pear-shaped or spherical bodies. Nematode bodies are transparent, unsegmented, and have no legs or other appendages. Plant parasitic nematodes have a hollow stylet or spear that is used to puncture holes in plant cells through which they withdraw nutrients from the plant.

Reproduction

Nematodes have well-developed reproductive systems that distinguish them as female and male nematodes. The females lay eggs, usually after fertilization by males but in some cases without fertilization. Many species lack males. Nematode eggs hatch into juveniles that resemble the adult nematodes but are smaller. Juveniles grow in size and each juvenile stage is terminated by a molt. All nematodes have four juvenile stages, with the first molt usually occurring in the egg. After the final molt, the nematodes differentiate into males and females, and the new females can produce fertile eggs in the presence or absence of males. One life cycle from egg to egg may be completed within 2–4 weeks in favorable weather, longer in cooler temperatures. In some nematodes, only the second-stage juvenile can infect a host plant, whereas in others all but the first juvenile and adult can infect. When the infective stages are produced, they must feed on a susceptible host plant or they will

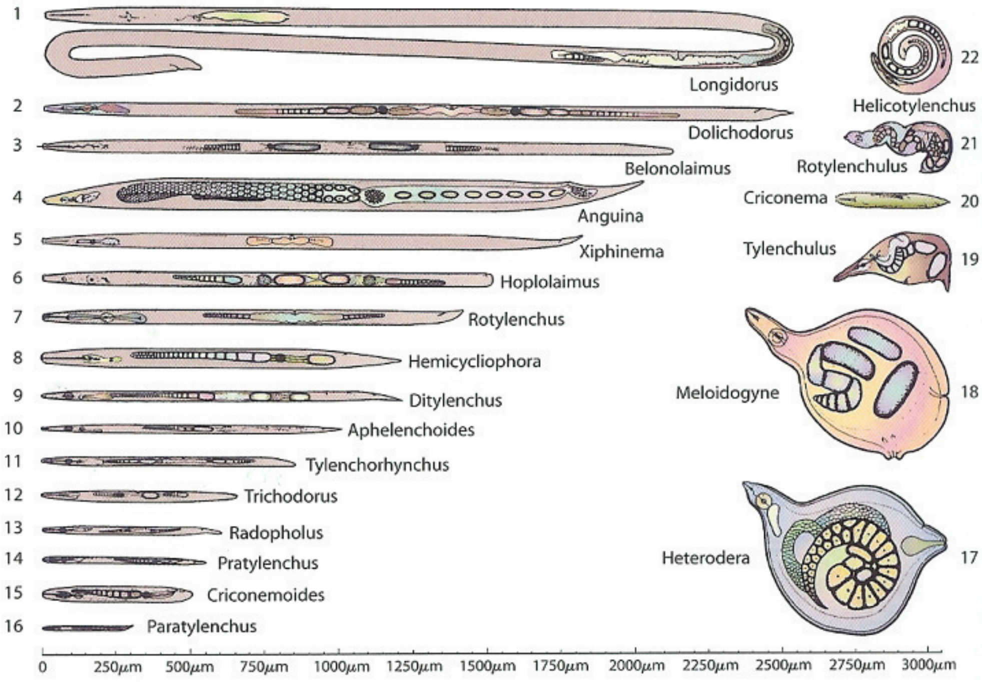
1
Longidorus
22
Helicotylenchus
2
Dolichodorus
21
3
Belonolaimus
Rotylenchulus
4
Criconema
20
Anguina
5
Xiphinema
Tylenchulus
19
6
Hoplolaimus
7
Rotylenchus
8
Hemicycliophora
9
Ditylenchus
Meloidogyne
18
10
Aphelenchoides
11
Tylenchorhynchus
12
Trichodorus
13
Radopholus
14
Pratylenchus
Heterodera
17
15
Criconemoides
16
Paratylenchus
0
250μm
500μm
750μm
1000μm
1250μm
1500μm
1750μm
2000μm
2250μm
2500μm
2750μm
3000μm

Figure 24 Diagram of shapes and sizes of most of the important plant nematodes. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 828. Burlington, MA: Elsevier/Academic Press.

starve to death. In some species, however, some juveniles may dry up and remain quiescent, or the eggs may remain dormant in the soil for years.

Classification

The plant pathogenic nematodes can be classified as follows (only a few important genera are listed):

Kingdom: Animalia

Order: Tylenchida

Genus: *Anguina*, seed-gall nematode; *Ditylenchus*, bulb and stem n.

Pratylenchus, lesion n. Radopholus, burrowing *n.*

Globodera, round cyst n. Heterodera, cyst.

Meloidogyne, root knot n.

Aphelenchoides, foliar n. Bursaphelenchus, pine wilt n.

Order: Dorylaemida

Genus: Longidorus, needle n. Xiphinema, dagger n.

Paratrichodorus, Trichodorus; stubby root nematodes.

Biflagellate Protozoa

To date, only a few diseases have been shown to be caused by plant pathogenic biflagellate protozoa. All of these diseases have been found to occur in the tropics of Central America. They include phloem necrosis of coffee, heart rot of coconut palm, sudden wilt or Marchitez sorpresiva of oil palm, wilt and decay of red ginger, and empty root of cassava. All these diseases seem to be caused by related protozoa of the genus *Phytomonas*. The protozoan diseases of plants, although rare, are severe on the infected plants, generally resulting in the collapse and death of the plants. The plant pathogenic protozoa invade and multiply in the phloem of diseased plants (**Figure 27**). They are mostly one-celled microscopic organisms that have flagella and typical nuclei.

Kingdom: Protozoa: (see also **Figures 2** and **3**)

Phylum: Euglenozoa, Order: Kinetoplastidae, Family: Trypanosomatidae, Genus: *Phytomonas*

Plant pathogenic biflagellate protozoa are transmitted from tree to tree by grafting and by insect vectors of the families Pentatomidae, Lygaeidae, and Coreidae.

Interactions of Pathogens, Plants, and Humans

Pathogens interact with plants and humans in a variety of ways. The main ways by which pathogens manage to survive, infect plants, and through the damage they cause to also affect humans, are also discussed briefly below.

Ecology, Dissemination, and Epidemiology of Plant Pathogens

Some plant pathogens (viruses, viroids, mollicutes, protozoa, parasitic higher plants, most nematodes, some obligate biotrophic oomycetes, the powdery mildew and the rust fungi, and the phloem- and xylem-inhabiting bacteria)

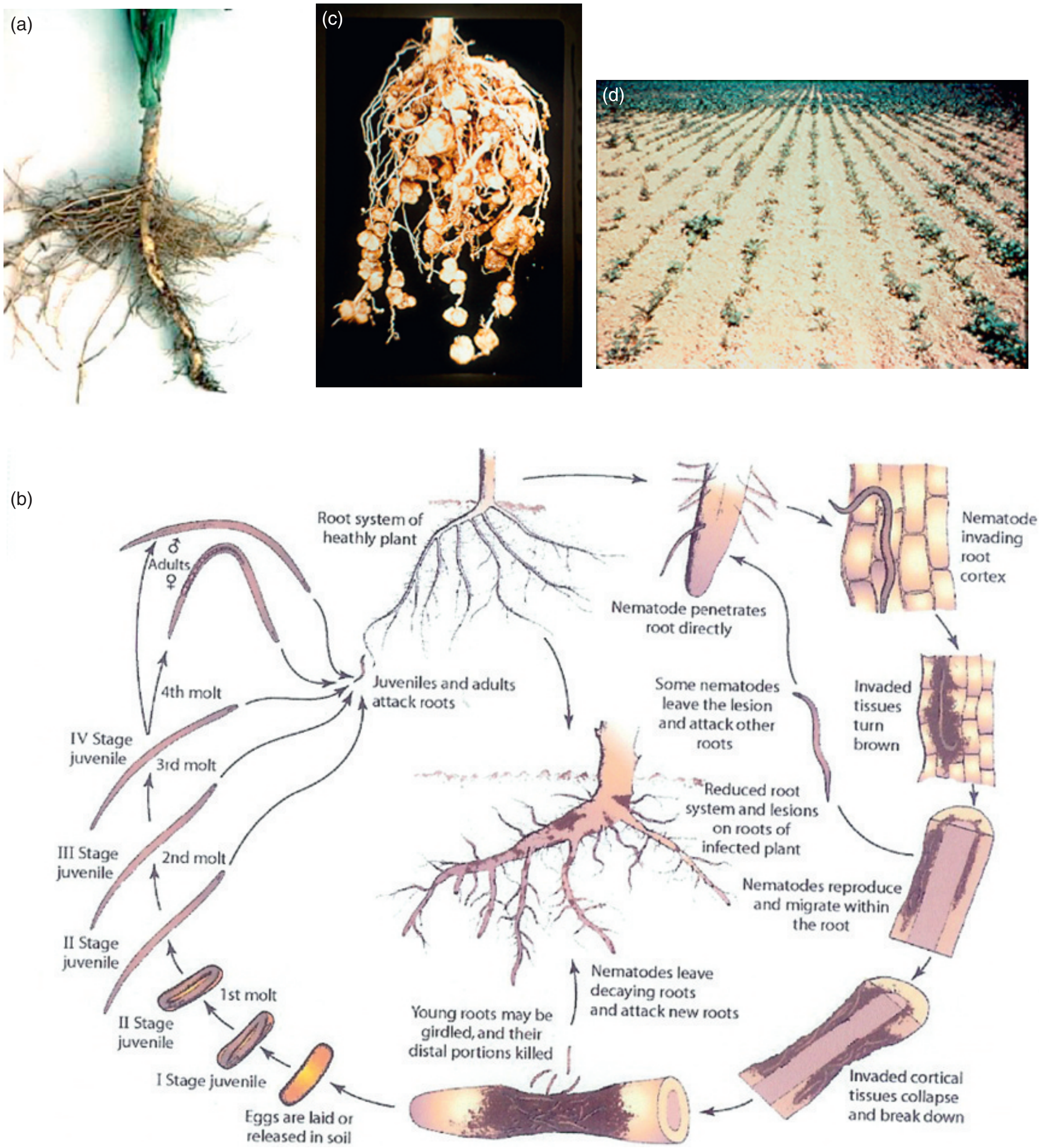
(a)
(c)
(d)
(b)
Root system of heathly plant
♂ Adults ♀
Nematode penetrates root directly
Nematode invading root cortex
4th molt
Juveniles and adults attack roots
Some nematodes leave the lesion and attack other roots
Invaded tissues turn brown
IV Stage juvenile
3rd molt
Reduced root system and lesions on roots of infected plant
III Stage juvenile
2nd molt
Nematodes reproduce and migrate within the root
II Stage juvenile
Nematodes leave decaying roots and attack new roots
1st molt
II Stage juvenile
Young roots may be girdled, and their distal portions killed
I Stage juvenile
Invaded cortical tissues collapse and break down
Eggs are laid or released in soil

Figure 25 (a) Damage of root of tobacco plant by the lesion nematode *Pratylenchus*. (b) The disease cycle of the lesion nematode. (c) Galls or root knots on roots of tomato plant infected with the root knot nematode (*Meloidogyne* sp.). (d) Sugar beet field in which a large area of sugar beet plants have been severely damaged or killed by the sugar beet cyst nematode, *Heterodera schachtii*. Photos: (a) and (c) courtesy of DW Dickson; (d), RJ Howard. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) p. 850, (b) p. 852, (c) p. 838, (d) p. 847.

spend all of their lives on their host plants. Most plant pathogenic fungi and bacteria live most of their lives in or in contact with their host plants, and only during periods of unfavorable weather they live in soil or in plant debris. Pathogens that live all of their lives in their hosts depend entirely on specific animal vectors, primarily certain insects, for movement from plant to plant, and for introduction into their hosts. Fungi produce different kinds of spores that can be carried by mild or strong winds to nearby or distant places. Most plant pathogenic bacteria do not produce spores and are carried as such by splashing rain, and so on. Almost all pathogens are carried in infected seed, transplants, tubers, and so on. When pathogens reach or exist in their hosts, they usually grow

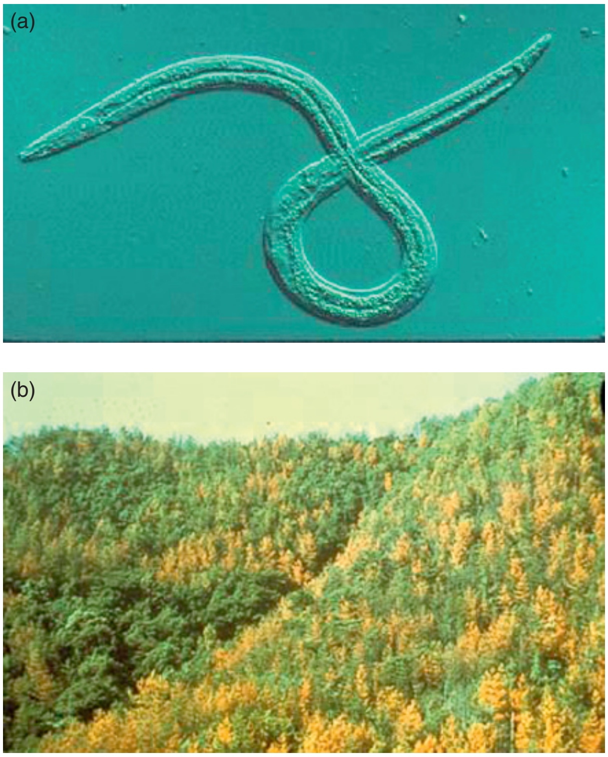

Figure 26 (a) Typical, although long, plant nematode, *Bursaphelenchus xylophilus*, causing wilt of pine trees. (b) Dead pine trees in a forest in Japan infected with the pine wilt nematode. Photos: (a) courtesy of RP Esser; (b), JJ Witcosky. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) and (b) p. 871).

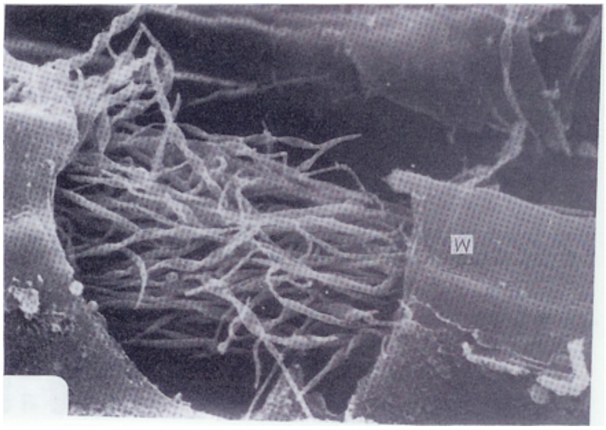

Figure 27 *Phytomonas* protozoa in a phloem sieve tube of root of oil palm tree affected with sudden wilt disease. W, plant cell wall. Photo courtesy of W deSousa. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn., p. 876. Burlington, MA: Elsevier/Academic Press.

inside host cells, but many fungi grow between parenchyma cells. The spores of fungi, nematodes, and some bacteria may come out of their hosts and be carried by air, water, or other means to other plans, whereas those living inside plants must be carried by their vectors.

Plant pathogens cause disease in plants when a virulent pathogen lands on a susceptible host plant, and the environmental conditions, primarily temperature and moisture, are favorable for the pathogen to be activated, germinate if it has to, and grow. Generally, high moisture favors most pathogens, especially bacteria and fungi, the latter because germination of their spores depends on the availability of moisture. Favorable temperatures and high moisture speed up infection, sporulation, spore release and new, secondary infections and, therefore, the number of disease cycles and severity of the disease during the season.

How Pathogens Infect Plants

Pathogens have evolved so that there is wide variability as to which kind of plant it can infect and which it cannot. Of the many kinds of plants, for example, corn, tomatoes, apple trees, each of them may be attacked by a hundred or more kinds of pathogens. For example, tomatoes may be attacked by 60 fungal pathogens, 15 viruses, 10 bacteria and mollicutes, and 15 nematodes, whereas corn may be attacked by 130 different pathogens, including 85 fungi, 8 bacteria and mollicutes, 20 viruses, and 17 nematodes. Perhaps none of the pathogens of corn are the same as the pathogens of tomato, or 2–3 pathogens, for example, some nematodes, may infect both plants. Hence, each pathogen is limited as to which plants can provide it with food. However, even if the pathogen (inoculum) has reached the plant (inoculation), there are still a number of steps that the pathogen must go through before the plant allows it to feed and to multiply.

Infection by fungi and bacteria

The mechanisms of infection of plants by pathogens and of defense of plants to infection are much better understood in diseases caused by fungi and some bacteria than in any of the other pathogens.

Once a pathogen comes in contact with a susceptible plant, and provided that there is enough moisture and the temperature is favorable, the sequence of the many active phases of the plant disease cycles begins. The pathogen must be able to adhere to the host, which the pathogen does by excreting sticky substances. If the pathogen is a fungal spore, or a bacterium, the latter and the spore must get ready to penetrate the host surface. Bacteria, and many fungi, enter plant tissues through wounds or through natural openings. Many of the fungi, however, can also enter the host plant through direct penetration of the plant epidermis. For these fungi to penetrate, the spore must germinate, so an entire new machinery must go to work to mobilize the inert spore into an active inoculum. The germinating spore produces a short germ tube on the surface of the plant and at the tip of the germ tube the fungus forms an appressorium, that is, a specialized feeding organ present in some organisms. The appressorium is oppressed and sticks to the plant surface on which it produces a penetration peg that exerts tremendous pressure on the epidermal cell it tries to penetrate. Finally, a thin infection hypha grows through the opening created by the penetration peg and the fine hypha resumes its normal diameter and absorbs nutrients from the plant.

Up to that moment of penetration by the infection hypha, the fungus spore provided genes for producing adhesive substances, enzymes, cytokinins, and so on, for the activation and germination of the spore, for producing the appressorium and then the penetration peg, which enabled the fungus to reach the food-laden contents of the plant cell. If the plant is susceptible to this race of the pathogen, the pathogen will produce enzymes for breaking down the substances that make up the plant cell wall, such as chitinases for chitin, cutinases for cutin, cellulases, pectinases, ligninases, and so on, to break down cellulose, pectins, and lignin, and enzymes for breaking down cell content substances, such as amylase for starch, proteases for proteins, and lipases for lipids and fats. This, of course, allows the pathogen to grow and multiply and, eventually, to produce spores on the plant surface.

In the meantime, through their enzymes and other toxic substances that they produce in the plant, and depending on the plant organs they infect, the pathogens may cause smaller necrotic (dead) spots or larger patches of plant tissue to die and rot, to shrink, or to show other symptoms. Many fungal and bacterial plant pathogens may also produce toxins that can kill tissues directly, or growth regulators that cause infected plant tissues to grow and produce galls rather than be destroyed and die.

On the other hand, if the plant is resistant to the pathogen, as soon as some molecule or factor (elicitor) of the pathogen is recognized by the plant, the defenses of the plant, both structural and chemical, preexisting or induced, are mobilized and the pathogen may become neutralized, and this leads to the death of the pathogen and to no plant infection.

Bacteria infect plants in a manner similar to that employed by fungi, except that bacteria enter plants through wounds or natural openings and, therefore, do not need or produce a germ tube or haustoria, but multiply and move about the host rather than grow into them.

Infection by pathogens surviving only in living cells

Plant viruses, viroids, mollicutes, xylem- or phloem-limited bacteria, protozoa, and nematodes, with just a few exceptions, enter plant cells either with the help of a specific vector such as a specific insect, mite, fungus, or nematode, which, with their mouth parts, pick up virus from infected plants and introduce it into healthy plants through the wounds they make while feeding, or they are brought into the host plant by pollen. Once a virus (or viroid) enters a plant cell, the virus sheds its protein coat and the naked nucleic acid (RNA or DNA) associates with appropriate cell organelles, primarily ribosomes, and also membranes, enzymes, and so on, which replicate the nucleic acid into hundreds of thousands or millions of copies. Soon, translation of part of the nucleic acid strand results in the production of molecules that are the subunits of the viral coat protein and these assemble with the nucleic acid strands and form the complete virus particle (a virion). In the process of virus replication and accumulation, the metabolism of the host plant is affected.

The defense mechanisms of the infected plant are activated, and if the defenses are effective, that is, if the plant is resistant, virus replication and movement may be stopped, the infected and a few surrounding cells may die, and no further infection develops beyond a tiny visible or invisible local lesion. If, however, the host defenses are ineffective or inadequate, the virus moves from cell to cell, replicating in each of them, and finally reaches the phloem sieve elements that make up part of the phloem veins. Once in the phloem, the virus is distributed throughout the plant and from the phloem cells it moves into adjacent parenchyma cells and to the other living cells in which it again replicates. Depending on the age and size of the plant, viruses can spread throughout the plant within a few days to a few weeks or months. Infected plants are often stunted, their leaves, shoots, and fruit become smaller, discolored, or malformed, yields are reduced, and with some virus–host combinations some plant parts or whole plants may be killed.

Nematodes: When an infective nematode juvenile or adult reaches the surface of a plant, it pierces the outer epidermal cell wall with its stylet, and secretes saliva into the cell, the contents of which are liquefied and absorbed through the stylet. Some nematodes are rapid feeders and feed only on epidermal cells, moving their stylet from one cell to the next without ever entering the plant. Others enter the plant parts and feed slower while inside the plant. Some of the latter become attached to one area of the plant and do not move. Some of the nematodes enter the plant, feed internally for varying lengths of time, and then exit the plant and move about freely. Depending on where the female of a particulate nematode species is feeding, she lays her eggs inside or outside the plant. When the eggs hatch, the new infective juveniles either cause further infections inside the plant or infect new plants. The mechanical injury caused by nematodes in the infected area, as well as the removal of nutrients from plants by nematodes, certainly has a detrimental effect on the plant. It is thought, however, that much greater damage by nematodes on plants is caused by the enzymes, growth regulators, and toxic compounds contained in the secretions of the nematodes into the plant and by the ports of entry for other pathogens (fungi and bacteria) created by nematodes on plant roots.

How Plants Defend Themselves against Pathogens

Because each pathogen can attack and infect only a few kinds of plants, that is, its own hosts, we presume that nonhost plants are resistant, indeed immune, to these pathogens. Also it is likely that all plants normally produce structures such as thick-walled epidermal cells, fewer or smaller stomates, and chemicals such as phenolic compounds. These structures or chemicals, by their presence in or around plant cells, act as the first mechanisms of plant defense against any pathogen that must overcome them to reach the interior of the cells for the food in it. In addition to such preexisting structural and chemical defenses, plants seem to be induced by the presence of the pathogen, to produce induced structural defenses such as cork layers, abscission layers, gums and tyloses, and biochemical defenses consisting of numerous biochemical reactions taking place in infected cells and tissues.

Indeed, the induced biochemical reactions play a major role in plant defense against pathogens. In the host–pathogen combinations that have been studied, as the pathogen comes in contact with a host plant, some of the molecules secreted by the pathogen, or released from host plant tissues by enzymes of the pathogen, react with receptor molecules present in host cells. The plant receptor molecules recognize such existing or induced plant molecules (elicitors) as harbingers of a pathogen attack and quickly trigger a cascade of defense reactions in the attacked and the surrounding cells.

One of the first products of defense reactions in the infected cell is the rapid and transient generation of activated oxygen species, which, in turn, triggers the hyperoxidation of plant cell membranes, which produce molecules toxic to the plant cell and to the pathogen. Such cells become dysfunctional and lead to cell collapse and death. The activated oxygen species are also involved in host defense reactions by activating the phenoloxidase enzymes that oxidize phenolic compounds into more toxic quinones. In addition, oxidation of membrane lipids produces several biologically active molecules, such as jasmonic acid, which is the precursor of the wound hormone traumatin, and, along with salicylic acid, appears to induce numerous protein changes and acts as a signal transducer of the defense reaction in plant–pathogen interactions. The affected plant cells continue to defend themselves by producing cell wall strengthening materials such as callose, glycoproteins such as extrensin, phenolic compounds, and so on. They further produce a variety of antimicrobial substances known as pathogenesis-related proteins, and a variety of secondary metabolic compounds, such as chlorogenic acid, caffeic acid, and ferulic acid. Some plants, upon infection with a pathogen, produce phytoalexins, which are toxic antimicrobial substances produced in appreciable amounts in plants only after stimulation of the plant by various types of pathogenic microorganisms or by chemical and mechanical injury. Besides, although the plant cells produce antimicrobial compounds aimed against the pathogen, the plants, in some diseases, defend themselves by detoxifying the toxins of the pathogen.

If the defense reactions are quick and effective, further growth and activities by a biotrophic pathogen are limited or stopped, and the infected cell collapses and dies. Because the pathogens can no longer attack and obtain food from such plants, such a plant is considered resistant to the pathogen. This type of defensive response by the plant, following recognition of a pathogen elicitor by the receptor of a plant cell, is known as the hypersensitive response (HR). Frequently, however, the term ‘HR’ is used to indicate that the host plant reacts to a particular pathogen by producing small local lesions and therefore is considered as resistant. Plant defenses against pathogens may be in the form of single or multiple, structural or chemical barriers, in the form of programmed plant cell death that stops further growth of biotrophic pathogens, or in activation of defense responses regulated by the salicylic acid-dependent pathway, or, more commonly, in a combination of various mechanisms of defense. Necrotrophic pathogens, which actually benefit from host cell death, are not limited by cell death and the associated salicylic acid-dependent defenses but rather by a different set of responses activated by jasmonic acid and ethylene signaling. If no defense reactions occur, or if they are ineffective or too slow, the plant becomes infected, and such a plant is considered susceptible to the pathogen. Generally, resistance in commercial crop plants can be, and usually is, the result of one or more resistance genes (R gene). In almost all wild plants, and in most cultivated ones, however, resistance in a plant to infection by one or more pathogens is the result of the expression of many plant genes controlling various defensive structural, chemical, and physiological characteristics of the plant.

Role of Genetics in Disease Initiation and Development

When a pathogen attacks a plant, it may not be able to infect it because the plant belongs to a taxonomic group outside the range of the pathogen and has genes for resistance for which the pathogen has no corresponding genes for virulence. This is called nonhost resistance. In another kind of resistance, the host may actually be susceptible to the pathogen but because it possesses genes that allow it to grow earlier or later than the time that the pathogen is present (disease escape), or allow it to grow and produce a crop in spite of it being infected with the pathogen (tolerance), this type of resistance is called apparent resistance. In most cases, however, plants are resistant to a pathogen because they possess genes for resistance (R genes) directed against avirulence genes of the pathogen (true resistance or race-specific, cultivar-specific, or gene-for-gene resistance).

Plant pathogens have genes for pathogenicity, which make them pathogens, and genes for virulence or avirulence, corresponding to genes for susceptibility or resistance in the host. This statement is probably the most straightforward statement about the role of genetics in disease and calls to mind that, for each gene for resistance in a plant, there is a gene for virulence in the pathogen and vice versa. Pathogens are pathogenic on some varieties of the host plant but not pathogenic on other varieties of the same host. Plants have genes for resistance, which produce the innumerable structural and biochemical substances that help them defend themselves, whereas pathogens have virulence genes that enable them to infect susceptible plants. Pathogens also have avirulence genes thought to code for molecules that, when recognized by specific receptor molecules, produced by resistance genes in the plant, act as elicitors of the host resistance response. Recognition of the avirulence gene product (elicitor) of the pathogen by the plant cell-produced defense response gene product (receptor molecule) sets in motion the production of a variety of structural compounds and biochemical reactions that lead to cell disruption and eventually to the death of the attacked plant cell and of the attacking pathogen. The death of the two combatants (attacking pathogen and cell of attacked plant) stops further development of infection and disease. In addition to the virulence/avirulence genes, several other types of genes seem to play important roles in the development and expression of disease.

When a resistant variety (A) is widely distributed for cultivation, within 2–4 years the variety loses its resistance to pathogen (a) and becomes susceptible. That variety, therefore, must be replaced with a new variety that contains a new gene for resistance (B) against the new gene for virulence (A) of the pathogen. Actually, the variety (A) did not lose any resistance. Instead, the pathogen, indeed all races of it, because they were excluded from the resistant new crop (A), was placed under extreme survival pressure and this led to the selection or appearance by mutation of a new variant of the pathogen individual(s) that carried the gene for virulence that enabled the pathogen to overcome or bypass the product of the gene for resistance (A). This new individual, being the only one with virulence gene (B), is unaffected by the old gene for resistance (A) and has all the plants with that gene to itself, without any competition from other pathogens. As a result, the new variant multiplies unimpeded and produces a new race of the pathogen, race (B) that can infect all plants of variety (A). The plant breeders, therefore, must produce a new plant that will contain a gene for resistance (B) that will stop the new pathogen. The easiest and best way to do that is by collecting individuals of the pathogen race (B) and inoculating as many new varieties or germ lines they have and hoping to find one or more plants that do not become infected with this race. This new variety or individual is propagated to produce the new variety (B) of the crop that will replace variety (A) in the field.

Effect of Pathogens on Plants, Crops, and Humans

Pathogens affect plants in some general ways regardless of some specific differences depending on the kind of plant, the kind of pathogen, and the prevailing weather conditions during the initiation and development of disease.

The first and main effect of pathogens on plants they infect is that they change the appearance of plants and produce the symptoms characteristic for each disease. Symptoms are almost always accompanied by reduced growth, productivity, and quality of the plants. It is the amount of yield that goes to the heart of the reasons plants are cultivated by humans. The extent of reduction is important not only because it reduces the expected income or profit but also because yield reduction as a result of disease results in hunger and starvation of humans and animals affected by the loss. Yield losses may be insignificant (2–3%), low (10–30%), large (40–60%), or complete (100%). Such losses are generally avoided and absent in areas and countries with advanced education and economies and where humans have the knowledge and the resources to buy foodstuffs from countries in which the disease was not as destructive that year.

In advanced countries, severe disease losses may be of little concern to a few people, who may have to go to the bank and borrow money to buy from other places quantities of the crop needed for consumption and for seed for the next year. In poor areas and poor, underdeveloped countries, however, severe losses of a staple crop may affect the survival of humans, animals, and industries that may exist and flourish in a large area or country. In poor countries, people have no knowledge of the cause of a plant disease and how to control it, no access to resistant varieties or to fungicides, if available, or collateral to borrow money from a bank and to find quantities of the crop in other countries. Also, because of lack of knowledge, a catastrophic epidemic of a plant disease occurring 1 year is likely to be repeated in subsequent years because of the large amount of pathogen inoculum that survives in the field and that can cause disease again the following year. Therefore, undernourishment, extreme suffering from hunger, and devastating famines caused by plant diseases are commonplace in poor, little-developed countries, and some of them, such as the Irish famine of 1845 and 1846, caused by the destruction of the potato crop by the late blight oomycete *P. infestans*, and the Bengal famine caused by the destruction of rice by the leaf spot fungus *Cochliobolus miyabeanus*.

When plants are attacked by pathogens frequently and repeatedly until they can no longer survive the attacks of these pathogens, there may be effects on whole ecosystems. For example, repeated infections of wheat and of forage grasses by the rust, smut, or ergot fungi reduce the amounts of food not only to humans but also for wild animals dependent on such plants for their survival. Although such repeated attacks occur on plants of all shapes and sizes, we are most aware of those occurring on trees because they are easier to see and to follow the happenings to them by a particular pathogen. The best known of these are the destruction and near extinction of the American chestnut by the chestnut blight disease, caused by the fungus *Cryphonectria parasitica*, in the Northeastern United States and Canada; the destruction and near extinction of the American elm by the Dutch elm disease caused by the fungus *Ophiostoma nova-ulmi*; the near extinction of the coconut palm tree in Florida and the Caribbean basin by a phytoplasma mollicute that still has not been named or classified, and so on. In many cases, the survival of some plants is impossible in areas that have been invaded by a pathogen, which then becomes a permanent inhabitant of the area, as happened in Central American banana fields showing infection with the Panama disease, caused by the fungus *Fusarium lycopersici if.* sp.*cubense* that became unfit for the production of bananas after the field became contaminated and thoroughly infested with the pathogen. This effect reduces the amount of land available for each crop that is susceptible to that pathogen and forces growers to plant crops other than the one that is more productive and profitable.

Plants infected with certain pathogens, for example, rye or wheat, infected with the ergot fungus, *Claviceps purpurea*, become unfit for human or animal consumption because the fungus produces fruiting structures, called ergots, that replace several of the seeds in each head. Ergots (**Figure** 7(**a**)) contain a large number of toxic alkaloids and other compounds, and humans or animals eating even a small amount of ergots become severely diseased because the contents of the ergots damage internal organs, such as the kidneys, the nervous system, and the circulatory systems, and result in severe painful diseases characterized by gangrene of the extremities of affected individuals.

Certain fungi infect mature, harvested seeds of grains and legumes and also bread, hay, or plant products such as before or after they are placed in storage. Such infections produce in the seeds a variety of toxic substances, called mycotoxins, although they do not produce any characteristic structures as do plants infected with the ergot fungus. Peanuts and corn seem to be the seeds affected most frequently and most severely by these fungi, especially if they are damaged and when the weather is very humid. Mycotoxins are extremely toxic and cause severe hemorrhage, serious diseases of the nervous and circulatory systems, damage to one or several of the internal organs, vomiting, rejection of food, and others. The most common fungi that produce mycotoxins in infected plant seeds and products are *Aspergillus*, which produces aflatoxins, ochratoxins, and so on; *Fusarium*, which produces trichothecins (vomitoxin), zearalenone, and fumonicins; *Penicillium*, which produces patulin, roquefortin, and so on, and several others.

A few fungal pathogens of grasses grow inside the plants (endophytes), and although these pathogens do not seem to cause apparent diseases on their hosts, they produce toxic compounds that cause severe diseases in the wild and domestic animals that eat the plants.

All plant diseases cause some financial losses because of reduction in the quantity and quality of the plants and plant products they infect. Such losses become increasingly more important as the amounts of losses increases and as the size of the area affected by a disease increases. The amount or percentage of losses is, of course, particularly important to the growers whose crop was affected by the disease – and some growers, who were fortunate for their crop to escape the disease, may actually profit by the higher price they get as a result of reduced supplies and increased prices for the crop. In spite of the few who may profit from a plant disease, the vast majority of times, everybody, growers and consumers alike, lose from it. The reasons for this is that the costs for growing such a crop will necessarily increase for every grower in subsequent years because they may have to replace the susceptible variety with a resistant one that may not be as productive or as desirable, may not be as profitable, and may be susceptible to another pathogen present in the area; besides, it will take time for all growers to acquire seed of the new variety. Also, the grower may need to change methods and equipment for cultivation, harvesting, and storage and transportation of the new variety or new crop, may need to build refrigeration, and so on. Finally, the grower may need to use pesticides to manage or control the disease, and this will add more costs for purchase, storage, and application of pesticides, plus the environmental costs of applying pesticides to the crop. Besides, for many diseases, such as those caused by viruses, phloem- and xylem-limited fungi, bacteria, mollicutes, and so on, no pesticides are available, and therefore this method of control with pesticides often is not available.

Detection and Identification of Plant Pathogens

Although some pathogens, such as the parasitic higher plants, are big and can be identified by their structure, most pathogens are small and must be looked under the microscope (nematodes, fungi, bacteria), for example, or even underthe electron microscope (viruses, bacteria, protozoa), to even begin getting close to a correct identification. Generally speaking, pathogens are difficult to identify.

For detection and identification of plant pathogens it is fastest when one reexamines the symptoms, amount Attempt to culture pathogens. Different and modern techniques are used. Although microscope use is routine, and access to electron microscope is increasing, the use of modern serological tests and DNA/RNA can provide a faster, more accurate, and more dependable result.

Most fungi and nematodes can be identified by examination of whole nematodes under the microscope and fungi by examination of the kinds of their spores and fruiting bodies.

Viruses and viroids are identified by their symptoms and by inoculating other plants and comparing them with the symptoms shown by other inoculated plants; checking epidermal cells for characteristic cellular inclusions; exposing viruses to different virus vectors; and, wherever available, most certainly serological tests and tests involving DNA and RNA.

The value of quick identification of a pathogen that may cause a potentially serious disease has increased greatly in recent years both because it has been proven repeatedly that plant diseases, like human and animal diseases, cause disproportionately more damage to crops when detection and diagnosis are late and slow and because of the political realities and threats to the security of our food supply. Extreme prolonged droughts, attack of plants by diseases, insects, and so on; political interference over large agricultural areas in the past few years have already resulted in frequent hunger and several famines affecting the poor people inhabiting these areas.

The need for rapid and correct diagnoses of the causes of plant diseases, insects, and so on; has lead the University of Florida, at Gainesville, FL, to create a new program of graduate study called the Doctor of Plant Medicine (DPM) Program, The DPM program leads to a professional Doctorate degree. The main purpose of the DPM is to train students at the graduate/doctoral level, on how to diagnose and how to control any kind of disease, injury or damage caused by any biotic (insects, mites, weeds, plant pathogens, birds, etc.) or abiotic factors (such as nutritional deficiencies, cold temperatures, air and soil pollutants). The 4-year program accepted its first 15 students in 2000 and by 2007; it had graduated nearly 40 Doctorates in Plant Medicine.

Management and Control of Plant Diseases

Some of the most common and most effective measures for management or control of plant diseases, regardless of the kind of pathogen, include the following:

1. Exclusion of the pathogen from a field by planting pathogen-free seed, seedlings, tubers, and so on.
2. Eradication of infected parts or entire plants, and of possible alternate hosts of the pathogen.
3. Improving cultural practices so that they support better growth of the host while impeding growth of the pathogen. These include appropriate fertilization, watering, pruning, thinning, temperature manipulation, and so on.
4. Planting resistant varieties whenever possible and available. One should always plant varieties resistant to the particular pathogen, and preferably to more than one pathogen. As many varieties are resistant to only one gene for resistance, one should expect that new races of the pathogen will soon bypass or overcome the resistance gene and therefore must continually produce varieties resistant to each of the pathogen races that carry different genes for resistance. Breeding varieties resistant to disease has received a tremendous impetus by employing the tools and methodology of genetic engineering and biotechnology.
5. Management and control with chemicals. Plant pathogenic fungi and bacteria are sensitive to numerous chemicals (fungi to fungicides, bacteria to bactericides). Fungicides (and bactericides), applied on the surface of plant foliage, blossoms, stems, and fruits, protect, to a certain extent, these organs from infection. Some fungicides are absorbed by the plant roots, and are distributed systemically through the plant, stopping infections wherever they may be initiated. There are no fungicides or other chemicals effective against viruses, viroids, or any of the other pathogens limited to the xylem or phloem of the plant.
6. Biological control. Some plant pathogenic fungi and bacteria are inhibited in their growth and ability to cause disease when exposed to certain antagonistic or parasitic microorganisms present in the vicinity (**Figure 28**). This is called biological control. Unfortunately, for only about 1% of the diseases have materials and methods been developed, but even with these, control is not satisfactory.

In addition, inoculating plants with mild strains of a virus protects them from infection by severe strains of the same virus. In the past several years, successful control of plant viruses has been achieved by genetically engineering plants to carry and express the coat protein and other genes of the virus, which makes the plants resistant to subsequent infection by that virus.

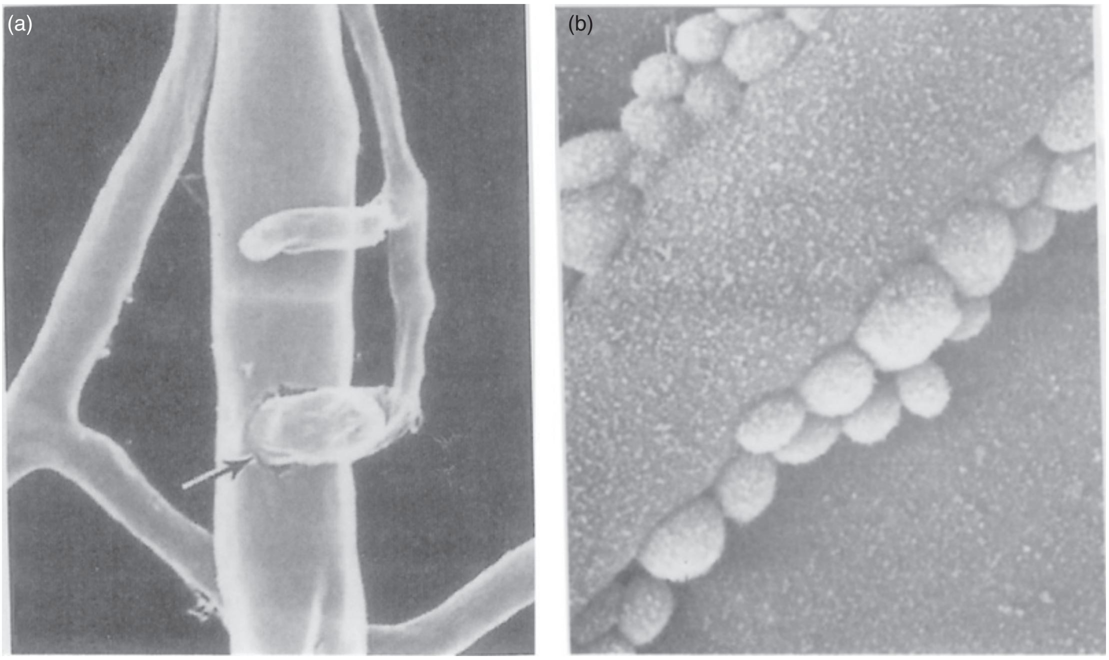

Figure 28 Biological control of certain plant pathogenic oomycetes and fungi with (a) other oomycetes or fungi that penetrate and feed on the first. (b) A plant pathogenic fungus (*Botrytis* sp.) is attacked by a yeast fungus (not pathogenic on plants). Photos: (a) courtesy of R Baker; (b), P Wisniwski. Reproduced from Agrios GN (2005) *Plant Pathology*, 5th edn. Burlington, MA: Elsevier/Academic Press, (a) and (b) p. 306.

Nematodes also benefit from the above, but when nematodes are present in a field, they can be managed primarily by planting resistant plant varieties and by applying pre- or postplant nematicides. Several cultural practices, such as plowing the soil before planting, expose the soil to the sun and dries it up and causes reduction of the numbers of nematodes present.

Conclusion

Plant pathogens, with a couple of minor exceptions, are microorganisms that belong to the same taxonomic groups, that is, bacteria, viruses, fungi, protozoa, and nematodes, which include the pathogens that cause disease in humans and animals. Each species of plants appears to be attacked by about 100 kinds of pathogens. Plant pathogenic fungi and bacteria live most of the time within their plant hosts and the rest of the time in the soil. The other pathogens live only in their plant hosts. Plant pathogens cause disease in plants and cause losses in food and other necessary items. The losses may be light or very severe, sometimes destroying all the plants and causing hunger, starvation, and famines, whereas in other cases they result in extinction of entire species of plants.

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Plasmids, Bacterial

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Defining Statement
Impact of Plasmid Research on Molecular Biology
Plasmid Structures and DNA Synthesis
Resolution and Distribution of Newly Replicated Plasmid DNA
Horizontal Plasmid Transfer by Conjugation
Further Reading

Glossary

biofilm A spatially organized community of microorganisms associated with a surface.

centromere The DNA segment of a replicon that is associated with spindle fibers and involved in DNA segregation.

cis From Latin for 'on this side of'.

DNA replication Duplication of the genome to make two copies of it.

helicase An enzyme that separates the DNA double helix into single strands.

integron A gene-capture system found in plasmids, chromosomes, and transposons that requires a recombinase enzyme (integrase) and a proximal recombination attachment site for incorporation into the genome.

microcosm A small enclosed part of a habitat.

origin of replication (ori) The site at which DNA replication starts.

polymerization The process of joining identical or similar subunits to make DNA, RNA, or proteins.

relaxase A site-specific topoisomerase that removes superhelical twists from DNA.

selfish DNA DNA whose only function is self-preservation (see also Further Reading).

Single-strand binding proteins (SSB) Proteins that facilitate replication by coating single-strand DNA, thereby preventing complementary regions from pairing with each other.

topoisomerase An enzyme that introduces or removes superhelical twists in the DNA.

trans From Latin for 'on the opposite side of'.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
BHR	broad host range
dso	double-strand *ori*
HGT	horizontal gene transfer
HR	homologous recombination
ICP	interon-containing plasmid
IHF	integration host factor
Inc	incompatibility
Mpf	mating pair formation apparatus
ori	origin of replication
oriT	origin of (DNA) transfer
PCD	programmed cell death
RCR	rolling circle replication
RE	restriction enzymes
Rep	replication proteins
RM	restriction–modification
SSB	single-strand binding proteins
ssi	single-strand initiation
sso	single-strand *ori*
TIVSS	type IV secretion system

Defining Statement

The focus of the article is on plasmids that establish themselves in bacteria. Strategies by which plasmids are reproduced, maintained, and transferred are described. The broader and interrelated issues of how plasmids generate a horizontal gene pool by recruiting genes from different environments, including genes for antibiotic resistance, are also discussed.

Impact of Plasmid Research on Molecular Biology

Bacteria are the most abundant and diverse forms of life on Earth and they play host to a vast assortment of extrachromosomal genetic elements. Joshua Lederberg originally proposed the word 'plasmid' as a generic term for any extrachromosomal hereditary determinant, a broader definition than is commonly used today. However, past and recent discoveries do point to common properties of certain bacteriophage and plasmids. Both can use very similar mechanisms for replicating, maintaining, and partitioning their genomes, and both can be evicted from a cell.

From the outset, plasmid research profoundly contributed to the development of modern molecular biology as summarized in **Figure 1**. Bacterial plasmids are exemplary subjects for study, being conveniently dissected, reassembled, and introduced into various hosts. Their versatility and power make them eminently worthy of our attention. Unraveling the complexities of plasmids relied upon genetics to identify the genes (proteins), biochemistry to determine their function, and microscopy to observe the conformations of single DNA molecules *in vitro* and the behavior of plasmid communities *in vivo*. The monumental task of elucidating the various shapes of DNA and how they impact replication, transcription, and recombination benefited immeasurably from the ease with which plasmids can be isolated and genetically altered.

Plasmids are selfish DNA that constitute a burden for the bacterial host cell. Their size can range from 2 to 500 kb and a given strain may contain several distinct plasmids. As a result, plasmids can commandeer a sizeable fraction of a cell's resources, at times providing little to no demonstrable benefit. For example, very few detectable phenotypic differences were observed under laboratory conditions after a wild type *Bacillus megatherium* strain was cured of its seven plasmids, which comprised 11% of the cellular DNA. How is it, then, that plasmids are so successful in colonizing bacterial communities and why are plasmid-free cell lines so infrequent among bacteria isolated from nature?

The DNA of plasmids and their hosts have coevolved a complex network of control mechanisms assuring a highly effective symbiotic relationship, albeit a forced one.

Plasmid Structures and DNA Synthesis

All circular and linear plasmids are double-strand, antiparallel DNA helices. When circular plasmids are isolated from bacterial cells, the entire double helix is 'stressed', which can lead to a change in the actual number of base pairs per helix turn. Alternately, this stress can cause regular spatial deformations of the helix axis. In either event, the axis of the double helix then forms a helix of a higher order. It is this deformation of the helix axis in closed circular DNA that gave rise to the interchangeably used terms 'superhelicity' and 'supercoiling'. Supercoiling is a form of energy that can be stored in DNA molecules and used in various DNA transactions. As shown in **Figure 2**, supercoiled DNA is more compact in comparison to relaxed -nicked- circles. Several unusual DNA conformations can be stabilized by supercoiling and some well-characterized examples include cruciforms, R-loops, and open complexes, all of which have specific sequence requirements. Many proteins that modify superhelicity

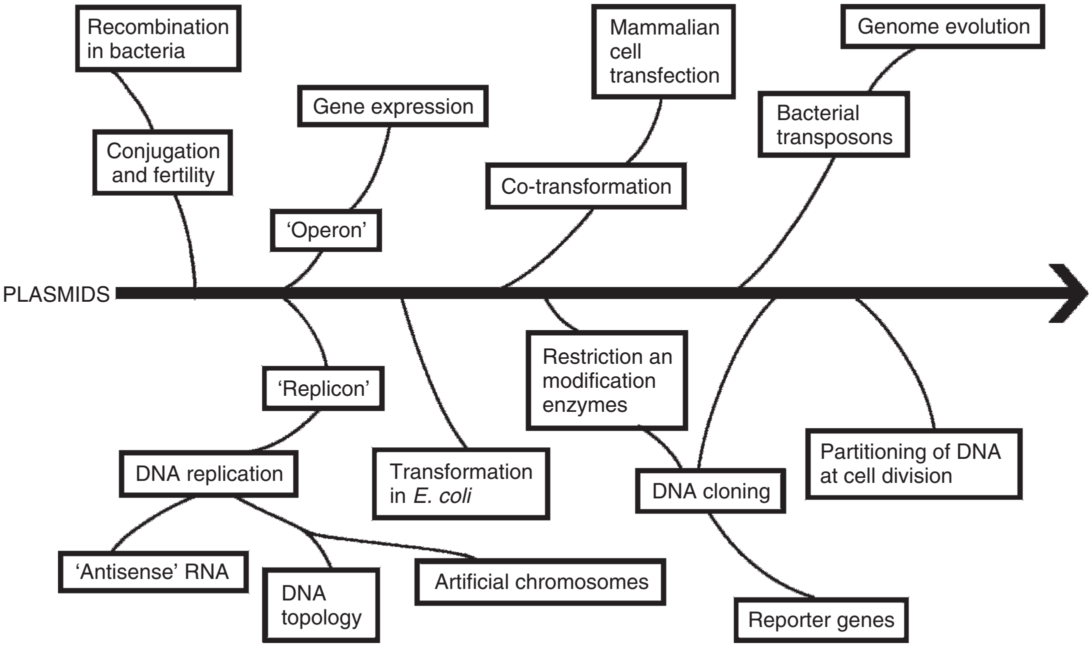
Recombination in bacteria
Conjugation and fertility
Gene expression
'Operon'
Mammalian cell transfection
Co-transformation
Genome evolution
Bacterial transposons
PLASMIDS
'Replicon'
DNA replication
'Antisense' RNA
DNA topology
Artificial chromosomes
Transformation in *E. coli*
Restriction an modification enzymes
DNA cloning
Reporter genes
Partitioning of DNA at cell division

Figure 1 Summary of some of the major contributions to bacterial genetics that have resulted from the study of plasmids. Reprinted from Elsevier, vol. 135, Cohen SN (1993) Bacterial plasmids: Their extraordinary contribution to molecular genetics. *Gene* 67–76. © 1993, with permission from Elsevier and the author.

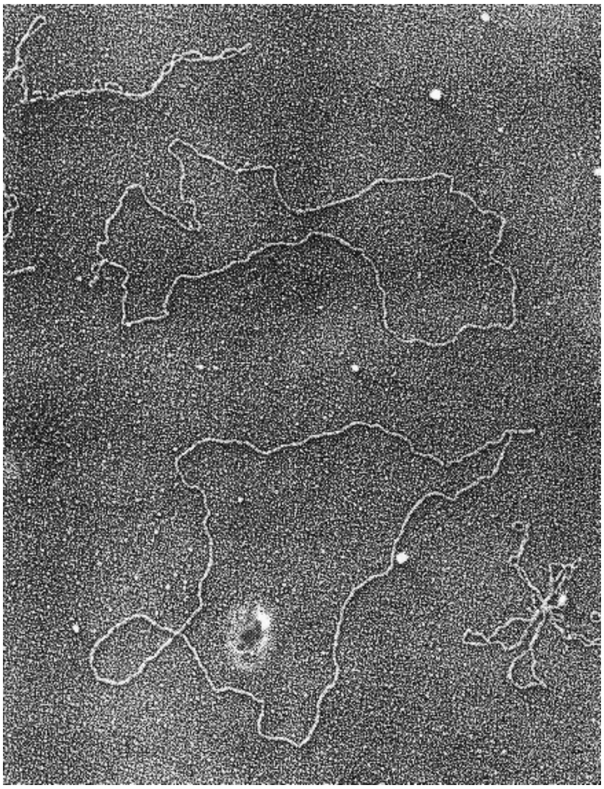

Figure 2 Electron microscope images of RI DNA. Predominant circular species appear as light images on dark background. The species in the top left and the bottom right corners represent superhelical plasmid DNA while two molecules in the center represent the relaxed form. Samples were prepared by a specialized technique that 'thickens' DNA and thereby amplifies the visual impression of DNA versus the background. Reprinted by permission from Macmillan Publishers Ltd: *Nature*. Cohen SN and Miller CA (1969) Multiple molecular species of circular R-factor DNA isolated from *Escherichia coli*. *Nature* 224: 1273–1277. © 1969.

catalytically (e.g., topoisomerases) or by simply binding to DNA and constraining superhelicity (e.g., integration host factor (IHF) and HU) exist. The known topoisomerases (Topo) that alter supercoiling include Topo I, DNA gyrase, Topo III, and Topo IV. Topo I and Topo III break 'one' strand of the DNA duplex resulting in their classification as 'type I' enzymes. The type II enzymes DNA gyrase and Topo IV are related to one another and, as their classification suggests, they break both strands of the DNA simultaneously. In almost all cases, isolated plasmid DNA displays 'negative superhelicity' (DNA replication). Synthesizing the ends of linear plasmids (i.e., telomeres) bucks this trend, however, since it is stimulated by positive supercoiling.

The process of DNA replication influences and responds to the superhelicity of the plasmid molecule. Initiation of plasmid DNA replication typically occurs asynchronously with respect to the bacterial cell cycle, unlike initiation at the chromosome's *oriC* (Chromosome replication and segregation). Irrespective of their target (plasmid or chromosome), all bacterial DNA polymerases require a 3′-OH group for initiating the synthesis of the leading DNA strand. This occurs via elongation that extends from one of three sources: an RNA primer (R-loop), a nick in one of the two strands of the double helix, or an amino acid of a protein covalently bound to the DNA. During synthesis of the lagging strand, many RNA primers are made. Eventually, the primer for the leading strand and the multiple RNA primers for the lagging strand are removed and the gaps are filled. DNA polymerases, multisubunit complexes by themselves, associate with many other proteins to form a molecular machine called the replisome. In some bacteria the replisome assembles at the cell midpoint or the predivision site. It was proposed that in Gram-positive bacteria with low GC content, two different polymerases might be used to replicate DNA (plasmids and chromosomes), one specializing in leading strand synthesis and a second that synthesizes lagging DNA strands. In Gram-negative bacteria, a single polymerase functions in both capacities. The replisome operates on single-strand template DNA created by the binding/activity of DNA helix-destabilizing proteins.

Unit of Replication: The Replicon

Units of replication called replicons comprise unique DNA segments that are indispensable for the plasmids (and chromosomes) that contain them. The reason is that the *ori* sequence from which replication 'originates' is embedded within each replicon. Interestingly, an *ori* of a circular replicon has been converted into a linear replicon by adding telomeres. Conversely, the *ori*s from linear plasmids have been similarly used to drive the replication of circular vectors. These results suggest that linear and circular replicons diverged from a common progenitor. Although a single replicon with a single *ori* is necessary and sufficient for the propagation of most plasmids, an additional level of complexity is found in a subset of plasmids that contain multiple *ori*s. Studies of plasmid replicons have revealed that an *ori* must be distinguished from the rest of the DNA and it is the only site where the frequency of replication is regulated. Once replication begins, DNA is threaded through the machinery at the fairly steady rate of about 1000 bp/s. 'Termination' of DNA synthesis occurs either at the *ori* (for unidirectional replication) or at a site called *ter* (for bidirectional replication) where the replication fork is disassembled. In linear plasmids, a different strategy for terminating replication is employed at the telomeres, which is described later.

To understand the components of the *ori* and the dynamics controlling its activity, an extraordinary effort has been made to isolate *ori*s from a variety of naturally occurring plasmids. Typical analyses of plasmid replication functions employ constructs called 'basic' or 'minimal' replicons. These are defined as the minimal portion of a plasmid that replicates with a copy number characteristic of the parent replicon. Minimal replicons are cryptic plasmids; they encode no function other than replication. Hence, to assess an *ori*'s ability to facilitate controlled replication, a selectable marker such as antibiotic resistance is often added (cloned) into these self-replicating DNA molecules. The recombinant plasmids that are made by this process confer antibiotic resistance to their host without posing risks to the environment because their construction and use are strictly regulated and confined to research laboratories. In fact, genetic markers of various types and combinations can be added to a plasmid replicon as long as the aforementioned rules are adhered to.

As might be expected from its complicated role, the *ori* coordinates multiple molecular interactions. Hence, it is somewhat surprising how simple some *ori*s are, with host-encoded RNA polymerase being the sole machine responsible for producing a preprimer. More often, before the replisome is assembled and the replication fork is launched, various proteins move in and sometimes out of the *ori* in a controlled sequential pathway to provide for the regulated initiation of DNA replication. Typically, one or many copies of a plasmid-encoded *ori*-specific protein bind to the *ori* and change its structure in a supercoiling-dependent process. DNA sequences to which these replication proteins (Rep) bind tend to be reiterated, thus earning the name 'iterons', and plasmids that carry them are called iteron-containing plasmids (ICPs). The presence of Rep-binding iterons is a hallmark, not only of many prokaryotic plasmid *ori*s, but of chromosomal, viral (phage), and eukaryotic *ori*s as well. Several well-characterized Rep proteins are known to bend the *ori* DNA when they bind to target sequences, be they singular cognate binding sites or iterons. Moreover, besides containing Rep-binding sites, some *ori*s contain multiple binding sites for a variety of proteins of which the most prominent are RNA polymerase, DnaA, IHF, HU, Fis, and H-NS. Each of these proteins is known to constrain supercoiling and some bend or even kink DNA upon binding. The distortions they produce lead to considerable changes in the DNA structure, and the resulting patterns of protein–protein interactions are needed to facilitate replication (**Figure 3**).

Examples of Replication *ori*s and Mechanisms of Their Activation

Circular plasmids are classified as belonging to one of three broad categories based on their mode of replication, which can be thought of in terms of their signature replication intermediates when visualized by electron microscopy. The terms that have been coined for two of these replication modes are particularly descriptive of these intermediates. Replicating theta-mode plasmids produce structures resembling the Greek letter theta, 'θ' (**Figure 4**). Rolling circle-mode plasmids, sometimes referred to as sigma mode for the Greek σ, appear as circles extruding linear product, giving the appearance of yarn rolling off a spinning wheel. The third replication

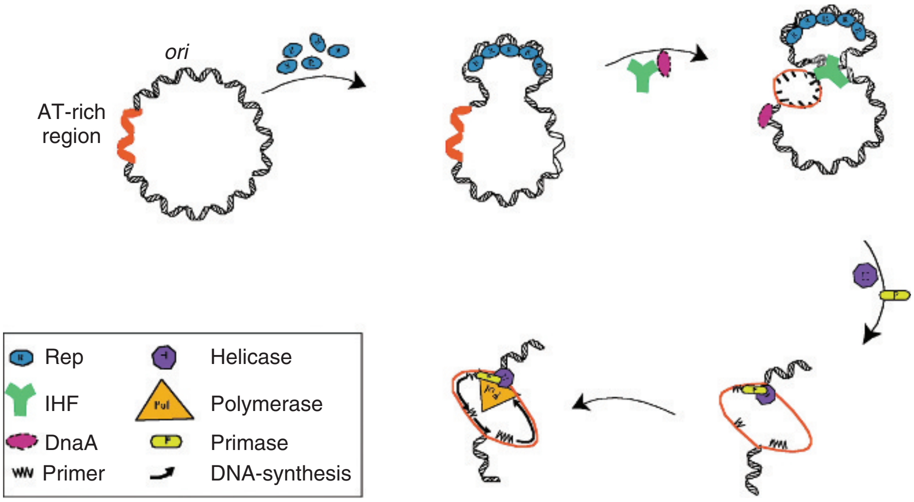

Figure 3 Replication steps – a model. The replication initiator protein (Rep) recognizes the origin of replication (*ori*) and induces a conformational change in the plasmid (e.g., DNA bending). Then, Rep protein, with or without host proteins engagement with their binding sites (IHF/HU, DnaA), triggers strand separation in an AT-rich segment of the DNA. This single-strand region is then targeted for the loading of DNA helicase and primase. DNA helicase will further unwind the DNA helix while primase will start synthesizing short RNA molecules that serve as primers for the initiation of DNA synthesis by 'sliding' DNA polymerase. Reproduced from Krüger R, Rakowski SA, and Filutowicz M (2004) Participating elements in the replication of iteron-containing plasmids (ICPs). In Funnell BE and Phillips GJ (eds.) *Plasmid Biology*, ch. 2, pp. 25–45. Washington, DC: American Society for Microbiology. © 2004, with permission from ASM Press.

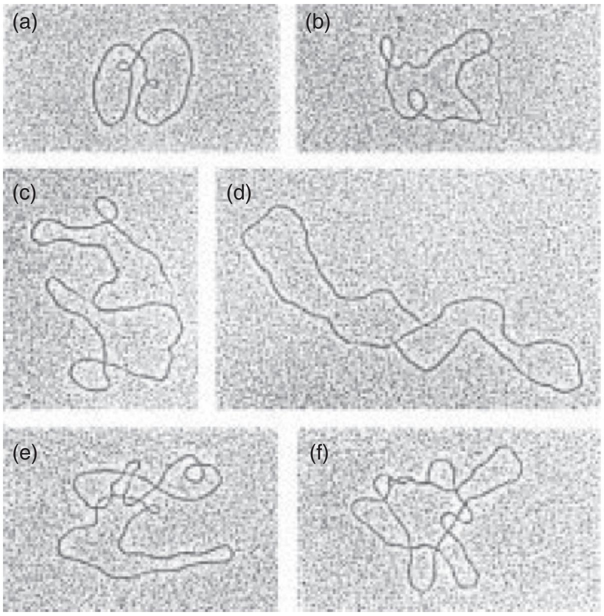

Figure 4 Electron micrograph of plasmid replication products synthesized *in vitro*: (a) Double-strand circular template DNA, (b) D-loop molecule, (c) and (d) Theta-type replicative intermediates containing two branch points and two double-strand daughter segments. (e) and (f) Catenated daughter molecules. To enhance picture resolution, the template and replicative intermediates were prepared for microscopy by a technique that 'relaxes' DNA. If not treated in this way, all samples would resemble the supercoiled molecules shown in **Figure 2**. Reprinted from Elsevier, vol. 193, Levchenko I, Inman RB, and Filutowicz M (1997) Replication of the R6K γ origin in vitro: Dependence on wt π and hyperactive πS87N protein variants. *Gene* 97–103. © 1997, with permission from Elsevier.

mode, strand displacement, has perhaps a less catchy name but it still informs the imagination.

Plasmids replicating by the 'theta' mechanism

Theta-type plasmids are divided into several distinct subgroups and many of the previously mentioned ICPs fall, collectively, into a theta subgroup characterized by *ori*s with AT-rich regions. For these plasmids, melting *ori* DNA during open complex formation typically requires the concerted action of both the plasmid-encoded Rep protein and the host-encoded initiator, DnaA. These proteins are believed to promote localized DNA melting at their binding sites, but there are insufficient data to explain how this would destabilize the spatially separated AT-rich segments. There is not even a consensus as to the type of nucleoprotein structure that DNA-bound Rep produces. In some cases, Rep proteins appear to form a discrete nucleoprotein complex into which none or only a short stretch of DNA is incorporated. In other ICPs, however, iteron-containing DNA seems to wrap around Rep, but only if another host protein (HU or IHF) is present, suggesting that interactions between the proteins occur in association with *ori* DNA. In fact, IHF may also have a role in strand separation.

Although the recognition of *ori*s by Rep protein and auxiliary host factors is energy-independent, the DNA melting process requires energy. Given that DnaA possesses ATPase activity, one possible role for this host factor in initiation may be to generate energy for strand separation. Surprisingly, however, ATP hydrolysis is not obligatory for DnaA functioning in the melting of some *ori*s despite the fact that data support crucial roles for DnaA–ATP and DnaA–ADP complexes in the regulation of chromosomal replication. Interestingly, in the presence of ATP, some Reps can change the conformation of plasmid DNA without any additional factors, suggesting that the Rep protein, itself, can somehow perceive the presence of ATP (**Figure 5**). Once melting is complete, the replication process proceeds to the synthesis of an RNA primer and the loading of DNA helicase. ATP is required as a cofactor as well as a substrate for RNA primers and there must be energy input for the process to progress. In a supercoiled DNA molecule, conformational energy stored after open complex formation can be tapped and, additionally, ATP hydrolysis occurs during the helicase movement and topoisomerase activities that take place ahead of the replication fork (gyrase, mentioned earlier).

The mechanism employed for primer generation is an important distinction used to assign plasmids to subgroups of theta-mode replication. In the R-loop-type plasmids, RNA polymerase-driven transcription generates an RNA molecule that is complementary to the transcribed strand of the plasmid DNA. Recent studies of RNA polymerases show that the RNA transcript and the DNA exit through separate channels of the polymerase, but the displaced DNA strand is accessible for base-pairing. When the 5′ end of the RNA and the displaced DNA strand interact, a stable RNA–DNA heteroduplex (the R-loop) is formed at the *ori*. This is highly unusual. Typically, the product of transcription is messenger RNA, which is bound by ribosomes and translated into proteins. In the prototype plasmid ColE1, a primer precursor of approximately 550 bp, called RNAII, interacts with the displaced DNA strand as RNA polymerase transcribes the template DNA. For DNA polymerase (Pol I) to initiate replication, RNA degrading enzymes must process the nucleic acid heteroduplex to make an RNA–DNA junction that will be proficient in priming unidirectional DNA replication. This group of plasmids does not encode any protein that is essential for replication. Contrasting with this, ColE2-type plasmids encode a Rep that functions as a priming enzyme specific for the ColE2 *ori*. Such enzymes, called primases, are simply alternate forms of RNA polymerase that are dedicated to synthesize primers for DNA replication. In the case of ColE2, the Rep/primase generates a very short RNA

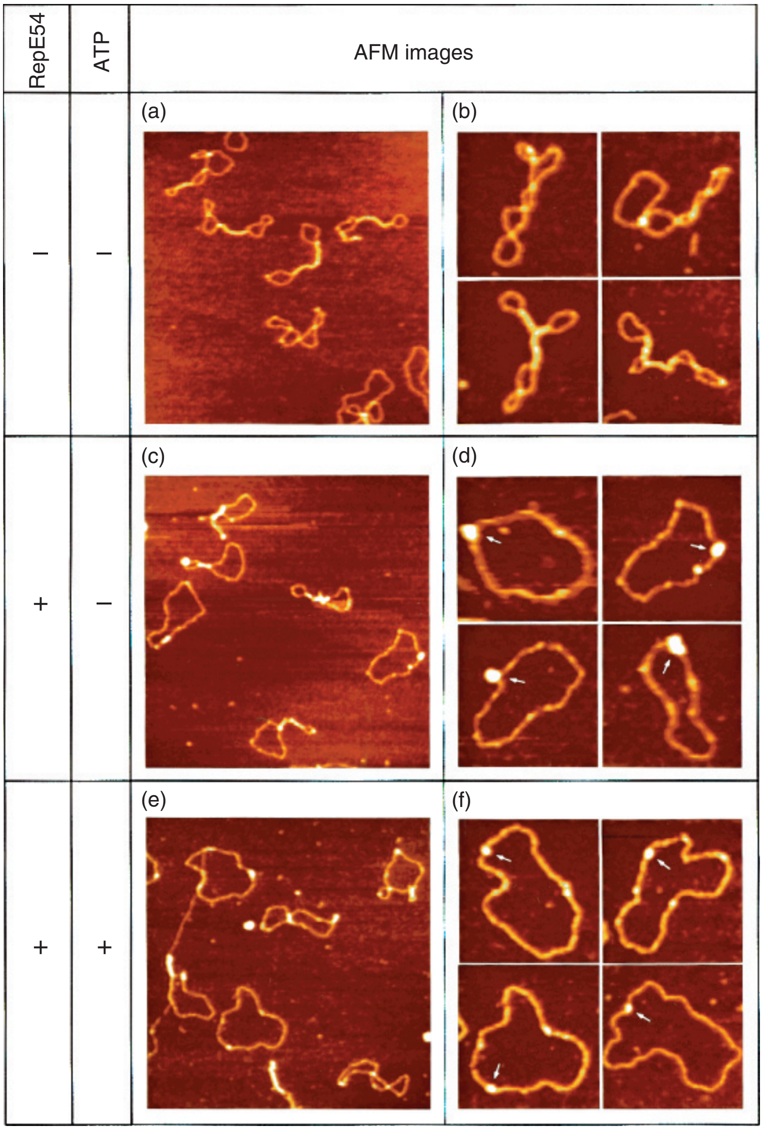

Figure 5 RepE54-induced relaxation of mini-F plasmid. Supercoiled plasmid and RepE54 were incubated in the absence or presence of ATP (at optimal amounts of all three reactants). Atomic force microscopy images of (a) and (b) supercoiled mini-F replicon DNA in the absence of RepE54, (c) and (d) relaxed plasmids by RepE54 binding in the absence of ATP, and (e) and (f) relaxed plasmids by RepE54 binding in the presence of ATP are shown. The images in f represent the relaxed plasmids only from the sample longer in 'apparent length'. Bound proteins are indicated with arrows. Image sizes are 1 × 1 μm (a, c, and e) and 300 × 300 nm (b, d, and f) (reproduced at 90% of original size). Reproduced from Yoshimura SH, Ohniwa RL, Sato MH, *et al*. (2000) DNA phase transition promoted by replication initiator. *Biochemistry* 39: 9139–9145. © 2000, with permission from the American Chemical Society.

primer, a scant 3 bases in length, to prepare for the synthesis of the plasmid's leading DNA strand.

Rolling circle replication

For plasmids that employ rolling circle replication (RCR) such as the PT181 family, the initiation step involves the recognition of the double-strand *ori* (*dso*) by the plasmid- and *dso*-specific Rep protein. Many *dso*s contain sequences that promote the formation of hairpin and cruciform structures in a supercoiling-dependent fashion. The binding of Rep to the *dso* is also known to enhance cruciform extrusion. Two enzymatic activities, nicking and strand closing, accompany the Rep protein's sequence-specific DNA-binding activity. In many RCR plasmids, a *nic* sequence is located in the loop of the hairpin and this sequence, in addition to the *dso*'s Rep-binding sequence, is required for plasmid replication. Reps are highly conserved among this family of plasmids, and sequence comparisons (of Reps and *dso*s) suggest that the hundreds of RCR plasmids may belong to only a few families.

Rep proteins encoded by RCR plasmids remain covalently attached to the 5′ end of the nick site via a conserved amino acid, tyrosine. Leading strand replication proceeds by extension from the free 3′ end of the nicked DNA until a complete round of synthesis leaves the parental portion of the leading strand fully displaced. Cleavage and rejoining reactions at the regenerated nick site, catalyzed by the Rep protein (using another tyrosine), result in a covalently closed, circular, double-strand DNA that contains the newly synthesized leading strand. Perhaps the requirement for two tyrosines explains why many Rep proteins of RCR plasmids function as dimers in which each subunit performs a different role. A common feature of the RCR initiators is that they promote only one round of leading-strand replication, a consequence of the inactivation of the tyrosine that Rep needs for initiation (an oligonucleotide is attached). To complete the replication process, the displaced leading strand is subsequently converted to double-strand DNA by using a single-strand *ori* (*sso*) and, solely, host-encoded proteins (**Figure 6**).

DNA replication during the process of conjugation

Conjugation, the self-controlled transfer of DNA, is an amazing property of some plasmids and a process of such significance that it warrants separate discussion later in this article (Conjugation, bacterial). The replication of DNA that occurs during conjugation is mechanistically very similar to RC plasmid replication, the major difference being that, in conjugation, the process commences in one cell but is completed in a different one. Conjugative plasmid replication begins with the relaxosome, which is an assemblage of proteins that processes the DNA at a site called the origin of (DNA) transfer (*oriT*). DNA relaxases are the key enzymes although they act, together, with additional accessory proteins. In all systems of self-transmissible and mobilizable plasmids studied so far (except in actinomyces), DNA cleavage is the consequence of a strand transfer reaction that involves the formation of a covalent DNA–relaxase intermediate. During conjugation, a unique plasmid DNA strand called the transfer (T) strand undergoes 5′-to-3′ directional transmission; thus, the relaxase-bound end of the DNA enters the recipient first. The 3′ end of this strand likely undergoes continuous extension by DNA polymerase in the donor cell, thereby generating a transfer intermediate that is longer than unit length and contains an internal *nic* site. The 5′-bound relaxase recognizes the site after it enters the recipient and mediates the recircularization of the DNA molecule by a reverse strand transfer reaction. In other words, a cleavage–rejoining reaction is catalyzed between the free 3′ OH end of the DNA and the covalently linked 5′ terminus. The initiation and termination steps in a round of transfer require different sequence features at *oriT*, consistent with the model that initiation involves negatively supercoiled, double-strand DNA whereas the termination reaction acts on single-strand DNA.

The T-strand that enters the recipient is 'parental' DNA. It is generally believed that replacement strand synthesis in the donor bacterium proceeds via a rolling-circle mechanism from the 3′ hydroxyl group exposed at *nic*. In the recipient cell, conversion to double-strand DNA occurs using a *sso* and host-encoded proteins. Another intriguing aspect of certain plasmids is that conjugative DNA replication and RC replication for copy number maintenance must

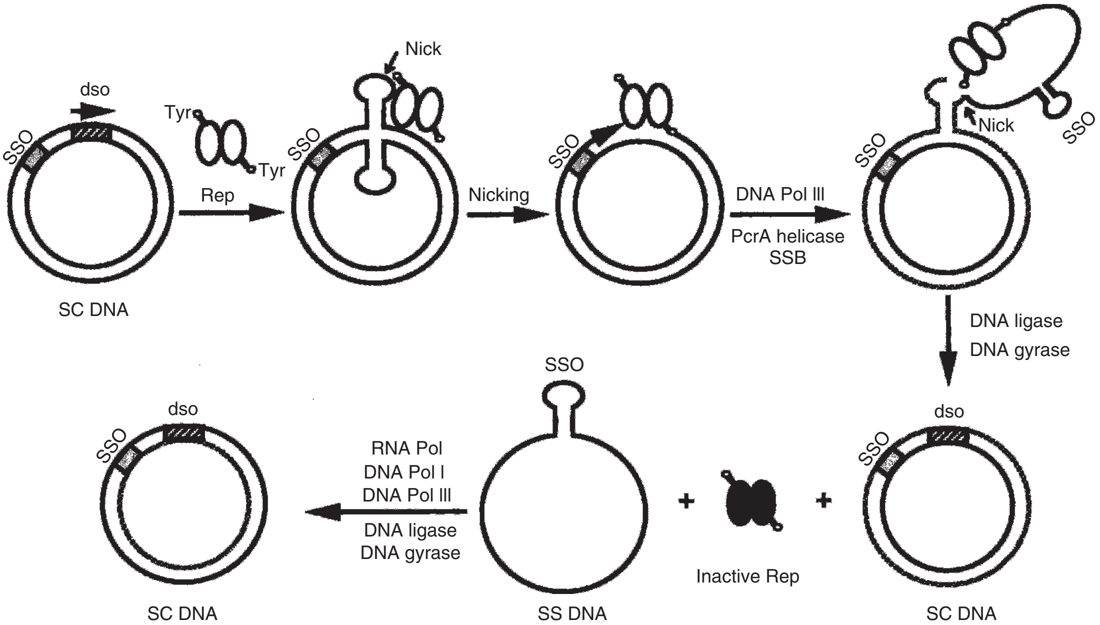

Figure 6 A model for plasmid RCR replication. See text for details. Reproduced from Khan SA (2004) Rolling-circle replication. In: Funnell BE and Phillips GJ (eds.) *Plasmid Biology*, ch. 4, pp. 63–78. Washington, DC: American Society for Microbiology. © 2004, with permission from ASM Press.

be strictly coordinated. After all, the initiation of DNA synthesis at *oriT* would lead to an immediate loss of superhelicity, thereby preventing cleavage at any other RCR type origin. Although the conjugative relaxases and Reps of RCR cleave DNA site- and strand-specifically, the relaxases have substantially higher affinity for the 3′-terminal region of the substrate DNA. This property of relaxases allows the superhelical state of the cleaved DNA to be maintained, which in turn allows the plasmids to exist stably as 'relaxosomes' without being impaired in other plasmid functions. As a result, relaxosomes can be present throughout the entire replication cycle, awaiting the cues that will trigger the release of the 3′ end. DNA cleavage by vegetative RCR initiators, in contrast, results in the spontaneous release of the 3′ terminus, which subsequently becomes available for elongation by host-encoded DNA polymerases.

Strand displacement: The IncQ family of replicons

The strand displacement mode of replication utilized by the RSF1010-like IncQ plasmids begins with a familiar scenario: Plasmid-encoded Rep proteins bind to iterons in the *ori*, which results in the melting of an adjacent AT-rich region. As a new twist, the opened DNA provides an entry point for a plasmid-encoded helicase whose activity exposes two single-strand initiation (*ssi*) sites. These sites are located on opposite DNA strands and are recognized by a plasmid-encoded primase. DNA replication proceeds by continuous extension of RNA primers by the host polymerase originating from a primed *ssi* site, and this results in the displacement of the nontemplate DNA strand. IncQ plasmids are exceptionally broad in their host ranges and their features will be discussed in greater detail in the section describing strategies used by plasmids for transferring themselves to plasmid-free cells.

Plasmid Replication: Regulation of Initiation Frequency

Every replicon's most basic function is to maintain itself at its characteristic, fixed, intracellular copy number. For some plasmids, the copy number can be as low as one per cell, whereas others can approach several dozen plasmid replicons per chromosomal equivalent. Ultimately, however, high copy and low copy plasmids adopt the survival strategy of controlling their rates of replication in accordance with the reproduction of their hosts. If the plasmid replicates 'too slowly' it will be lost from the bacterial population, but if it replicates 'too fast', cells will become 'intoxicated' by the excessive amplification of extrachromosomal DNA.

Functional assays called incompatibility (Inc) tests have been used to identify elements that control the copy number of plasmids. The name of the assay derives from another use of this powerful genetic tool, determining the functional relatedness of plasmids. An Inc^- phenotype often results from the inability of plasmids to coexist within the same cell over many generations because they share replication/copy number control elements; this eventually winds up excluding one of the plasmids from the cell. This assay led researchers to classify plasmids into several dozen Inc groups. By cloning fragments of minimal replicons into unrelated plasmids, Inc testing was adopted as a screen to identify factors and/or sequences that inhibit the replication of their plasmids of origin. This methodology demonstrated that, across the board, antisense RNA and iterons play a *trans*-acting regulatory role even though they encode no product; the RNA or DNA itself inhibits replication in a dosage-dependent fashion. The mechanism that senses the number of *ori*s per cell (*ori* counting) is dependent on the binding of antisense RNA or iterons to their targets, another RNA, or Rep protein. These targets were identified by using another major genetic approach used to dissect plasmid replication, the isolation of 'copy-up' mutants with elevated plasmid copy numbers. Copy-up mutations typically fall within the 'genes' encoding antisense RNA or Rep. It is noteworthy that certain copy-up mutations (singularly or in combination) have been found to cause a plasmid–host relationship to become self-destructive due to plasmid (over)replication.

ICPs: Regulation of replication in Rep–iteron systems

As previously discussed, an understanding of the control of replication in iteron-containing plasmids relies on a familiarity with the interactions between Rep protein and iterons, the dominant players in DNA helix melting at the *ori*. Helix melting is an early step of open complex formation, which is considered to be a prime target for elements in plasmid replication control. Insight into one possible mechanism for modulating *ori* activity was gained by the observation in one system (plasmid R6K) that precise deletion of mutant iterons can sometimes restore the function of a defective replication *ori*. It was hypothesized that continuous alignment of iterons invites near-neighbor contacts between the Rep molecules bound to them. More recent experiments have demonstrated cooperative iteron binding by Rep protein, which presumably has a positive influence on filling the *ori* with initiator even when concentrations of active Rep might be suboptimal. Decades of research have demonstrated that Rep and iterons are not only necessary for initiating ICP replication, but under certain conditions they act as negative regulatory elements as well. Our advanced understanding of replication control in ICPs owes itself to the fact that many Rep proteins and iterons exhibit sequence homologies and a still larger group (including eukaryotic counterparts) appears to be related on a structural level. This has allowed insights made in one model system to be applied to others. It has become evident that Rep proteins are characterized by structural flexibility, allowing them to participate in a wide range of regulatory communications (**Figure 7**). Crystal structure analyses and biochemical data have revealed that Rep monomers are modular in nature with one subdomain dedicated to DNA binding while the other can alternately be used for DNA binding or dimerization (**Figure 8**). In iteron-regulated plasmids, copy-up Rep mutations typically destabilize protein dimers and overwhelming evidence indicates that Rep monomers are the initiators of replication, not the dimers that often seem to predominate. In one or two systems, dimers can compete with monomers for iteron binding; however, most Rep dimers seem to lack iteron-binding activity. Intriguingly, a number of experiments have demonstrated that dimers inhibit replication and some of the data come from systems where dimers do not bind to iterons.

Whether or not dimeric versions of Rep bind iterons, they often play key regulatory roles in the autorepression of transcription and/or the inhibition of replication (**Figure 7**). One model for Rep-mediated inhibition of plasmid replication, known as handcuffing, has been proposed in variations that do and do not invoke direct DNA interaction by Rep dimers. The handcuffing model centers on three main postulates: An individual *ori* with Rep monomers bound to its cognate iterons is replication proficient. Rep-mediated coupling of two *ori*s by dimers blocks the initiation of replication for each of the participating plasmids. Finally, handcuffed structures fall apart and initiation potential is restored as cell volume increases, perhaps a result of dimers dissociating to yield two monomer-containing *oris* that are ready to recruit other replication components (Cyanobacterial toxins). There is ever-growing evidence to support handcuffing as a unifying mechanism of replication control among ICPs. Another model for negative replication control, called titration, also invokes Rep–iteron interactions but in an entirely different context. The underlying principle for titration rests on the notion that iterons could inhibit DNA replication by tying up Rep initiators in nucleoprotein complexes that are nonproductive for replication initiation. A straightforward prediction of the titration model is that increasing the concentration of the rate-limiting component should increase plasmid copy number. This prediction has been realized in ICP systems in which plasmid levels are proportional to Rep over a broad range of protein concentrations.

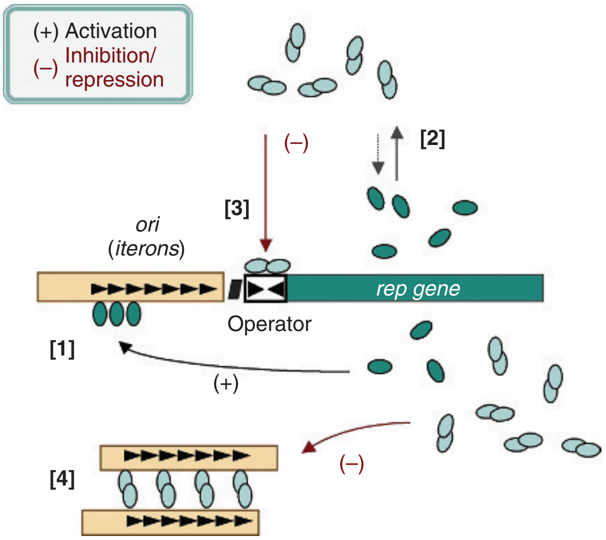

Figure 7 Two essential components of a 'minimal' R6K replicon are the γ *ori* and its cognate Rep protein, π, encoded by the *pir* gene. π monomers activate γ *ori* replication at low intracellular levels [1]. At elevated protein levels, π dimers form [2], autoregulating *pir* expression [3] and inhibiting replication. π dimers may use more than one mechanism to inhibit replication. Although handcuffing [4] is hypothesized to be a replication inhibitor in several plasmid systems, additional work established competitive protein–DNA binding as another viable mechanism for R6K (not shown). The monomeric initiator form of π and the dimeric replication inhibitor form bind iterons competitively and sequence specifically. Although π dimers are typically vastly more abundant, monomeric π has a couple of advantages, cooperativity and dual DNA binding domains.

Replication control by antisense RNAs

Data from a wide assortment of systems controlled by antisense RNA have revealed the formation of highly structured molecules that act via sequence complementarity on targets called sense RNAs. Copy-up mutations that destabilize antisense RNA or alter its interaction with the cognate sense RNA are known. Antisense RNA-dependent regulation of replication is achieved by a variety of mechanisms: (1) inhibition of RNA primer utilization by DNA polymerase, (2) inhibition of the translation of Rep protein or the leader peptide needed for efficient Rep translation, (3) attenuation of transcription to limit the availability of Rep. In some instances, the antisense RNAs act alone. In other cases, antisense RNAs act in concert with regulatory proteins that are either transcriptional repressors or RNA-binding proteins. The use of antisense RNA as a regulatory control element is further elaborated on in RNAs, small etc.

Resolution and Distribution of Newly Replicated Plasmid DNA

Resolution of the Products of Circular Plasmid Replication

Multimerization of circular replicons is a persistent problem in all recombination-proficient bacterial species. The best studied case of multimer formation is the dimerization of circular chromosomes, which is lethal when unresolved. A host-encoded recombination system, Xer,

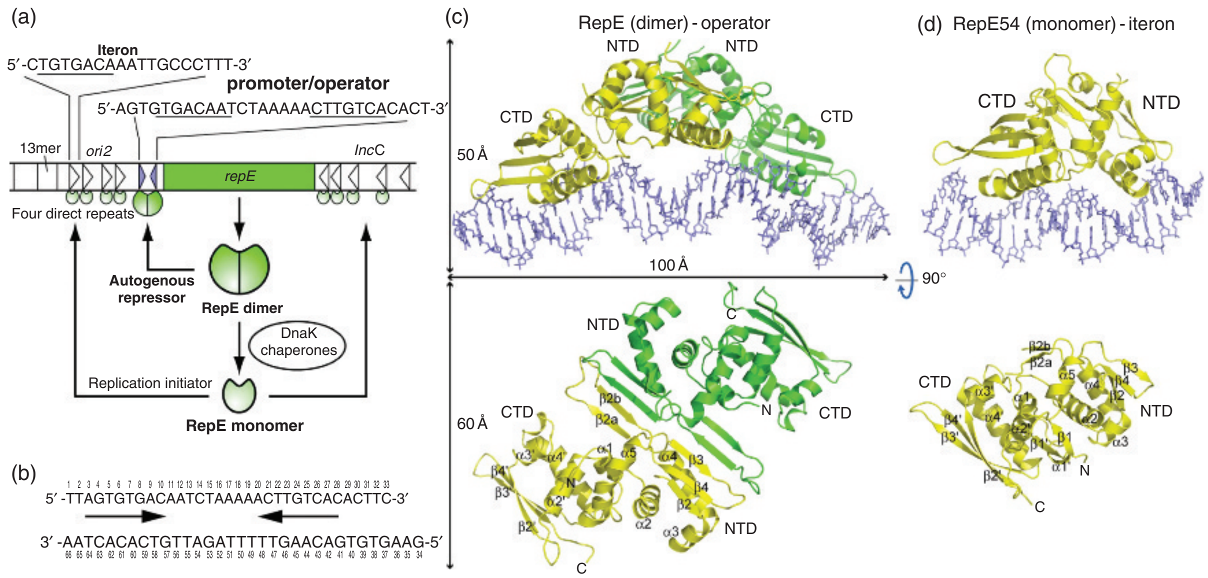
(a)
Iteron
5′-CTGTGACAAATTGCCCTTT-3′
promoter/operator
5′-AGTGTGACAATCTAAAAACTTGTCACACT-3′
13mer
ori2
repE
IncC
Four direct repeats
Autogenous repressor
RepE dimer
DnaK chaperones
Replication initiator
RepE monomer
(b)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
5′-TTAGTGTGACAATCTAAAAACTTGTCACACTTC-3′
3′-AATCACACTGTTAGATTTTTGAACAGTGTGAAG-5′
66 65 64 63 62 61 60 59 58 57 56 55 54 53 52 51 50 49 48 47 46 45 44 43 42 41 40 39 38 37 36 35 34
(c)
RepE (dimer) - operator
NTD
NTD
CTD
CTD
50 Å
100 Å
90°
60 Å
NTD
C
CTD
N
CTD
β2b
β2a
α1
α5
α4
β3
α3'
α4'
β4'
β3'
N
α2'
β2'
α2
β4
β2
α3
NTD
C
(d) RepE54 (monomer) - iteron
CTD
NTD
β2b
β2a
α5
α4
β3
β4
β2
CTD
α3'
α1
α4'
α2
NTD
β4'
β3'
α2'
β1'
β1
α3
α1'
N
β2'
C

Figure 8 Structures and functions of RepE. (a) Schematic representation of the functions of RepE initiator of plasmid F. (b) DNA sequence used for the cocrystallization of RepE with DNA. Arrows indicate the common 8 bp sequences shared by the *ori* iteron and the inverted half iterons present in the *repE* gene operator. (c) and (d) Two views (length and distance) of a RepE–operator DNA complex (c) and a RepE–iteron DNA complex. Each functional dimer is colored green (molecule A) or yellow (molecule B). The DNA models are omitted in the lower panels. The secondary structural elements of the RepE dimer are designated according to previously determined elements of the RepE54 structure. Reproduced from Nakamura A, Wada C, and Miki K (2007) Structural basis for regulation of bifunctional roles in replication initiator protein. *Proceedings of the National Academy of Sciences USA* 104: 18484–18489. © 2007, with permission from National Academy of Sciences, USA.

is required to convert chromosomal dimers into monomers and its high level of conservation among bacteria and archaea (with circular genomes) reflects its crucial role in chromosome segregation. Not surprisingly, ongoing sequencing projects reveal that most circular and linear plasmids contain one, and often multiple, site-specific resolvase genes.

Why is dimerization, and higher level multimerization, so problematic for a replicon? The formation of dimers affects replicon stability by lowering the number of segregation units at the time of cell division. As noted earlier, replication is controlled by 'origin counting', which means that a dimer counts as two plasmids for replication but only as a single unit for segregation. Imagine cell division advancing toward the production of two 'daughter' cells but with only one dimeric chromosome to be partitioned to them. Plasmids, even multicopy plasmids, are similarly ill-affected by the formation of multimers, which increases the frequency of plasmid loss. In fact, although it may seem counterintuitive, dimer formation poses a greater risk to the high copy number plasmids. These plasmids follow a 'random copy choice' mode of *ori* activation and, as a result, dimers replicate at twice the frequency of monomers. The replicative advantage of the multimers causes their rapid accumulation in the progeny of the cells in which they appeared. This phenomenon, called the 'dimer catastrophe', is responsible for the greater fraction of segregation defects in plasmid-bearing cells because it leads to the formation of a subpopulation that contains mostly multimers. Another serious disadvantage of multimer formation in circular, but not linear, plasmids is their high sensitivity to rearrangements caused by homologous recombination (HR) (**Figure 9**; Recombination, genetic). Certain recombination events among circular replicons result in the formation of a dimeric cointegrate molecule in which the two copies of the replicon are fused in a head-to-tail configuration. These events do not occur in a strain that is recombination deficient (e.g., *recA*) consistent with the view that the vast majority of plasmid dimers form by HR.

In addition to multimerization, circular plasmids are confronted with another obstacle to plasmid segregation, 'catenation'. The replication of both DNA strands of circular plasmids results in the formation of intercatenated structures in which the two sister double-strand DNA molecules remain interlinked (unseparable by pulling them apart). Sophisticated DNA-processing machines physically resolve catenanes and dimers (**Figures 4** and **9**). An enzyme mentioned earlier, type II topoisomerase, can promote decatenation by sequentially nicking and closing the two strands of the DNA backbone. Resolution of multimeric forms of circular plasmids (and chromosomes) is mediated by relatively simple molecular machines, termed site-specific recombinases or resolvases, that catalyze the essential DNA breakage and rejoining reactions. These enzymes are often plasmid-encoded with the recombinase

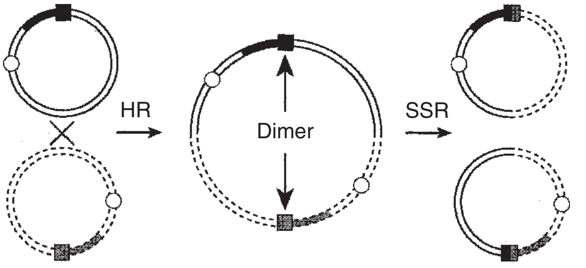

Figure 9 Formation and resolution of circular replicon dimers. Homologous recombination (HR) occurring during or after replication of a circular plasmid or chromosome produces a dimeric DNA molecule in which the two copies of the replicon are fused in a head-to-tail configuration. The dimer is converted into monomers by site-specific recombination between the duplicated copies of the replicon resolution site (colored in black and gray). The core recombination sites where the recombinase catalyzes the strand-exchange reaction are represented by squares. The adjacent colored regions are regulatory sequences that are often associated with the recombination site to control the recombination reaction. Circles represent the plasmid or chromosome replication origin. Reproduced from Hallet B, Vanhooff V, and Cornet F (2004) DNA site-specific resolution systems. In: Funnell BE and Phillips GJ (eds.) *Plasmid Biology*, ch.7, pp. 145–180. Washington, DC: American Society for Microbiology. © 2004, with permission from ASM Press.

gene and the target recombination site usually being associated side by side (i.e., linked loci). Resolvases fall into two major families of unrelated proteins that use different mechanisms to cleave and rejoin DNA molecules. These two groups of enzymes are now commonly referred to as the serine recombinase family and the tyrosine recombinase family according to the conserved residue that participates in the DNA cleavage–rejoining steps. Many plasmids, however, such as those of the ColE1 family, utilize the host-encoded Xer recombination system rather than encoding one of their own.

Recombination between directly repeated sites on a circular DNA molecule will resolve the molecule into two separate rings (**Figure 9**). Multimer resolution activity is totally independent of other cellular processes such as replication, allowing site-specific recombination to take place at any stage of the cell cycle. This is crucial to ensure efficient resolution of multimeric forms of circular replicons.

Termination and Resolution of Replication by Machines Assembled on Linear Plasmids

Linear plasmids and chromosomes have been identified in a number of widely divergent bacterial species and they generally retain the same features and mechanisms for replication initiation as their circular counterparts. In species that possess both linear and circular plasmids such as *Borellia burgdorferi*, a conserved mechanism for replication initiation appears to be the rule. Such replicons contain internal *ori*s from which replication proceeds bidirectionally toward the telomeres. In contrast to initiation, however, replication termination and the resolution of replicated plasmids are significantly different in linear and circular replicons. In circular plasmids replicating bidirectionally by the theta mechanism, the *ter* sequence is recognized by a contra-helicase called RTP that blocks the movement of the replicative helicase (DnaB), thereby promoting termination. The *ter* sequence signals the end point of replication for a molecule that has no physical ends. All linear replicons, whether eukaryotic, prokaryotic, or viral, are presented with a different challenge as replication nears completion: how to replicate the extreme 3′ ends of the DNA? Various mechanisms have evolved to solve this problem and are discussed below.

The ends of linear plasmids in bacteria fall into two main structural classes, telomeres with covalently closed hairpin ends and telomeres with unlinked DNA and protein-capped ends. Plasmids with hairpin ends can be described as continuous single strands of DNA that are self-complementary, so their structure is a double-strand linear molecule with direct linkages at both ends between the 5′ end of one strand and the 3′ end of the other. Due to the inherent stiffness of DNA, linear plasmids have loops composed of at least four unpaired nucleotides making the connection between the two strands, called a hairpin end. Plasmids containing hairpin telomeres versus free ends capped with terminal proteins require different termination mechanisms.

For the linear plasmids with closed hairpin ends, replication initiates from an internal origin and continues around the hairpin telomeres, resulting in a circular dimer. Processing of the circular dimer into two linear plasmids is accomplished by the activity of telomere resolvases that recognize specific sites (i.e., replicated telomeres) in the circular dimer formed after replication. The enzymes cleave the joined telomeres of replication intermediates, subsequently religating them to generate two daughter plasmids with hairpin ends (**Figure 10**). An inverted repeat is presumed to be the only sequence feature required for telomere resolvase to recognize and cleave joined telomeres. Given that the replication of this type of linear plasmid produces head-to-head circular dimers, it is not surprising that organisms harboring such replicons contain enzymes related to the tyrosine recombinases described in the previous section.

Evidence for an alternate method of processing the ends of linear plasmids can be seen in *Streptomyces* in which proteins are bound to the termini. As the bidirectional fork encounters the extreme 5′ end of the newly synthesized DNA strand, with its last RNA primer removed, a terminal patching mechanism is required to fill the remaining gap. The new DNA chain is approximately 300 nt short at the 5′ terminus, which leaves a single-strand 3′ overhang (**Figure 11**) Two mechanisms

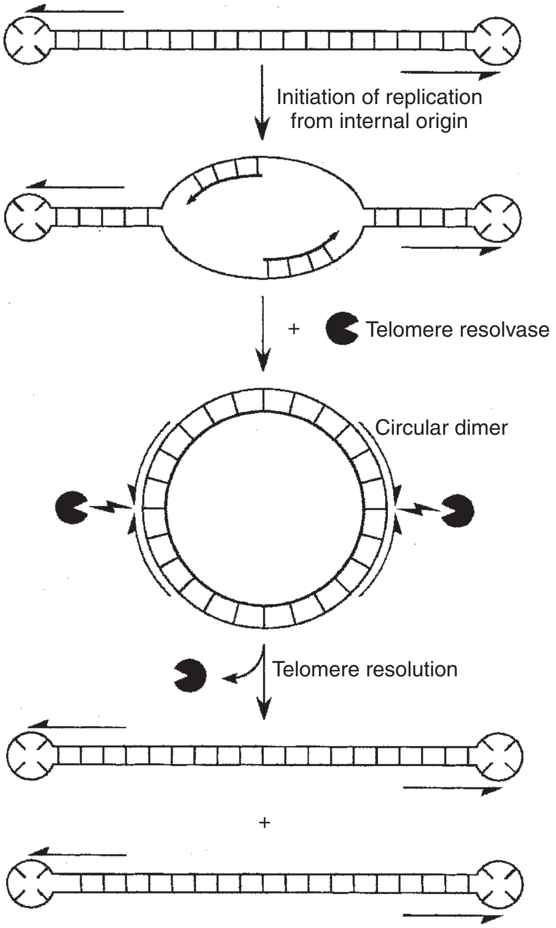

Figure 10 Model for replication of linear plasmids with covalently closed hairpin ends. Replication initiates from an internal origin and proceeds bidirectionally, producing a circular dimer intermediate with joined telomeric sequences producing inverted repeats (arrows). The replicated telomere sequence serves as a recognition site for the telomere resolvase (●), which cleaves both DNA strands and then joins opposite strands together to create two linear plasmids with covalently closed hairpin telomeres. For further details, see Further Reading. Reproduced from Stewart P, Rosa PA, and Tilly K (2004) Linear plasmids in bacteria: Common origins, uncommon ends. In: Funnell BE and Phillips GJ (eds.) *Plasmid Biology*, ch. 13, pp. 291–301. Washington, DC: American Society for Microbiology. © 2004, with permission from ASM Press.

have been proposed for filling in the missing nucleotides and each assigns roles for the terminal proteins, which are known to be essential although their exact roles in replication remain to be elucidated. In one model, the terminal protein functions as a protein primer to initiate gap filling. In a second model, the terminal protein nicks the template strand and attaches itself covalently to the 5′ end with subsequent displacement and gap filling by DNA polymerase. Whatever functions terminal proteins possess, they are likely conserved among this category of linear plasmids. The genes encoding terminal proteins have been found to lie adjacent to the plasmids' telomeres, and sequence analysis has revealed that the proteins are homologous.

Plasmid Segregation

Plasmid partitioning and plasmid replication are independent functions (Chromosome replication and segregation). This was perhaps best demonstrated by observations that several partitioning loci (coding and noncoding *par* 'genes') promote plasmid stability when cloned to different replicons striped of their own *par* systems. Partition machines also exert an Inc phenotype that is distinct from the replication-mediated Inc described earlier, the latter being the Inc that is traditionally used to classify replicons. As a result, two replicons that would be compatible based on their replication machinery will nonetheless be unable to stably coexist in the same cell if they are segregated by the same *par* system. This Inc mechanism derives from competition between identical partition systems on otherwise different plasmids and has been used to genetically dissect partition modules and their components.

Elegant microscopic studies support the contention that Par machines prevent plasmid diffusion in the intracellular space by organizing DNA into communities called foci then actively distributing plasmids to each side of the cell division plane. By using fluorescence microscopy it has become possible to track segregating plasmid molecules. The components of *par* systems and the dynamics of segregating plasmid foci are reminiscent of the mitotic machinery of eukaryotic cells. Remarkably, plasmid molecules rely on just three essential components for their specific intracellular positioning and, thus, stable propagation. The first of these elements, the centromere, is required in *cis* for plasmid stability. Centromeres often contain one or more inverted repeats as recognition elements and they serve as the loading sites for the rest of the segregation machinery. In addition to the centromeres, two *trans*-acting Par proteins are required; one or both Par proteins usually autorepress their own expression. Adaptor proteins specifically recognize the centromeres and the energy-generating cytoskeletal ATPases (or GTPases) that move and attach plasmids to specific host locations. Plasmid partition systems are typically classified according to the nature of the cytoskeletal component they encode.

ParM systems: Actin-like ATPases

Our understanding of partitioning systems is most advanced for the *Escherichia coli* plasmid R1 that encodes ParM protein, an actin-like ATPase. As revealed by fluorescence microscopy, ParM assembles into transient and dynamic filaments that grow at similar rates at both ends and then depolymerize unidirectionally. Additional insights were gained when Par-mediated movement was reconstituted from purified components. By hydrolyzing

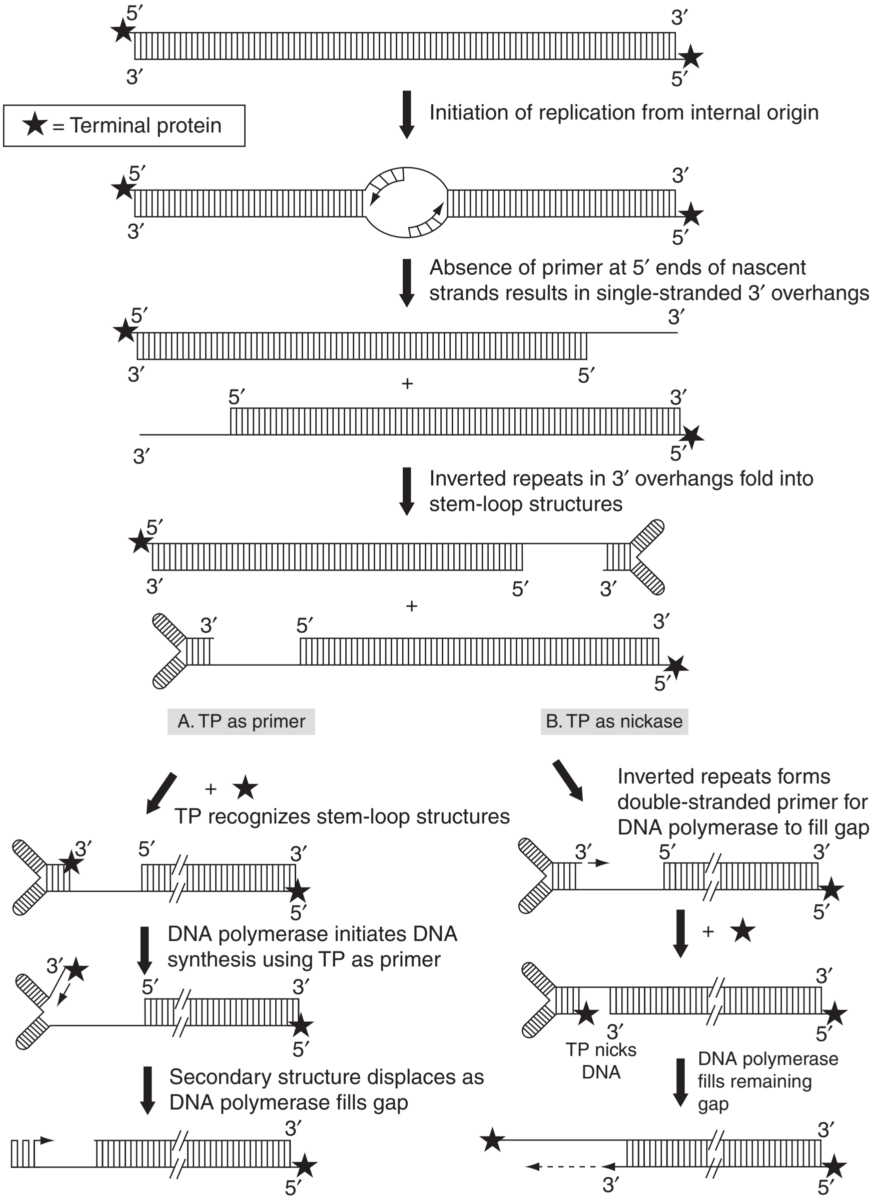
★ = Terminal protein
5′
3′
3′
5′
Initiation of replication from internal origin
Absence of primer at 5′ ends of nascent strands results in single-stranded 3′ overhangs
+
Inverted repeats in 3′ overhangs fold into stem-loop structures
A. TP as primer
B. TP as nickase
+ ★
TP recognizes stem-loop structures
DNA polymerase initiates DNA synthesis using TP as primer
Secondary structure displaces as DNA polymerase fills gap
Inverted repeats forms double-stranded primer for DNA polymerase to fill gap
+ ★
TP nicks DNA
DNA polymerase fills remaining gap

Figure 11 Model for the replication of linear plasmids with protein-capped ends. Bidirectional replication from an internal origin results in a gap at the 5′ end of the newly synthesized strand when the RNA primer is removed. Two general models for filling the gaps are depicted and are based on models for the replication of the linear chromosome of *Streptomyces* spp. Inverted repeat sequences of the single-stranded 3′ overhang fold together to form stem-loop structures. (a) The terminal protein (★) recognizes the complex secondary structure of the 3′ DNA strand and serves as a protein primer for DNA polymerase to initiate replication and fill the gaps. (b) The folded 3′ terminus forms the double-stranded primer necessary for DNA polymerase to initiate replication and fill the gap. Subsequently, the terminal protein binds and nicks the DNA near the beginning of the inverted repeat regions. DNA polymerase then proceeds in a 5′ to 3′ direction from the original template strand and fills the remaining gap. For variations on these models, see Further Reading. Reproduced from Philip S, Rosa PA, and Tilly K (2004) Linear plasmids in bacteria: Common origins, uncommon ends. In: Funnell BE and Phillips GJ (eds.) *Plasmid Biology*, ch. 13, pp. 291–301. Washington, DC: American Society for Microbiology. © 2004, with permission from ASM Press.

ATP, ParM polymerization was shown to facilitate the segregation of DNA complexes containing the adaptor protein (ParR) bound to centromeres (*parC*), thus providing strong evidence that these three elements of the *par* system are required and sufficient to mediate plasmid segregation. Researchers have been able to visualize the segregation *in vivo* and measurements reveal that it is a speedy process. ParM filaments grow by the insertion of monomers at the filament–plasmid junction and, like actin, the protein assembles head-to-tail into a polarized filament with distinguishable plus and minus ends. Plasmid DNA invariably localizes to opposite ends of a growing spindle, suggesting that polymerizing ParM actively pushes plasmids apart.

How do plasmid molecules manage to interact with opposite filament ends at the same time? A clue was provided by crystal structure data showing that ParR dimerizes to form a DNA-binding structure that further assembles into a helical (or ring-shaped) array with DNA-binding domains presented on the multimer's exterior. Additionally, electron microscopic analysis of ParR–*parC* complexes showed *parC*–DNA wrapped around the ParR protein scaffold. These findings suggest that the ParR–*parC* complex can encircle ParM filaments and slide along the polymer. As the ParR–*parC* complex has twofold symmetry, this interaction may occur in inverse orientations at opposite ends of the ParM filament, thus explaining the topological problem of how ParR–*parC* can interact with both ends of a polar filament.

In addition to the long ParM filaments, shorter ones appear to emanate from a single plasmid, which suggests a model (shown in **Figure 12**) that might explain how the *par* spindle works. ParM filaments form continuously throughout the cytoplasm but rapidly decay in the absence of stabilizing interactions with plasmid molecules. However, if a filament at one end of the cell becomes stabilized, it will 'search' the cytoplasm and only after it 'captures' a second plasmid will the filament extend into a pole-to-pole spindle. This is similar to the way in which microtubules extend from the eukaryotic spindle pole body during mitotic pro-metaphase, searching for chromosomes. Although bipolar stabilization of ParM filaments is favored when two plasmid copies are in close proximity, plasmid pairing itself is not required.

Other mechanisms for plasmid localization

The more widespread type I family of bacterial DNA segregation systems uses ATPases whose signature ATP-binding amino acid sequence is referred to as the Walker type. The cytoskeletal ParA proteins form filamentous structures that move through the cell in an oscillatory pattern. Like ParR of plasmid Rl, the DNA-binding adaptor proteins (ParB) of these systems serve as tethers between ParA and plasmid centromere sites (*parS*). Although the mechanism by which the ParA system functions is less well understood, it is equivalent to its ParM counterpart in stabilizing plasmid molecules and, in fact, manages to distribute multiple plasmids along the length of the cell. Yet another system has been recently discovered (called type III) that, quite unexpectedly, displays treadmilling behavior rather than dynamic instability. Despite the obvious functional similarities as intracellular transport machinery, no homology exists between the force-generating proteins or the DNA-binding adaptor proteins of the three types of partitioning systems. With few exceptions, the organization of *par* functions in linear plasmids is similar to those of circular replicons. The N15 linear replicon stands out as one of the odd balls. Its protein-encoding *par* loci are genetically unlinked from any centromere and it has palindromic sequences dispersed across the genome that function as centromeres. Overall, much remains to be elucidated concerning plasmid segregation mechanisms, but as more plasmids become sequenced and characterized, the plasmid segregation repertoire is likely to expand. This anticipated wealth of new data in combination with sophisticated fluorescence microscopy will lead to the advancement of this field.

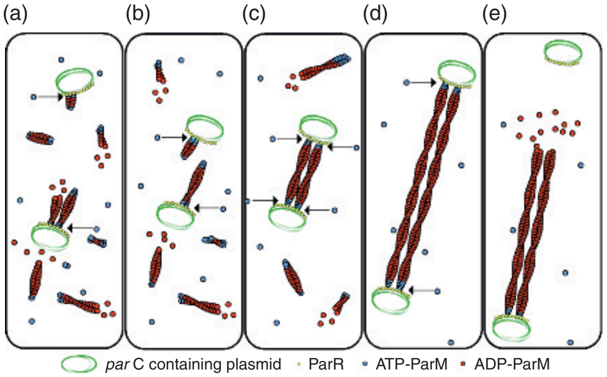

Figure 12 Molecular model of plasmid segregation by the RI *par* operon. (a) Nucleation of new filaments will happen throughout the cell. Filaments attached to one plasmid will search for a second plasmid. (b) Plasmids will diffuse around the cell until they get close enough to encounter each other. (c) When two plasmids come within close proximity, filaments will be bound at each end by a plasmid, forming a spindle. This will prevent the filaments from undergoing catastrophe. (d) As these stabilized filaments polymerize, the two plasmids will be forced to opposite poles. If the ends of a spindle run into the sides of the cell, it will be followed along the membrane to the ends of the cell. (e) After reaching a pole, pushing against both ends of the cell causes the filament to dissociate from the plasmid at one end and quickly depolymerize. Reproduced from *The Journal of Cell Biology*, 2007, 179: 1059–1066. © 2007 The Rockefeller University Press.

Until late 1990 it had been widely held that plasmid molecules are scattered throughout the cell. Thus, it came as a surprise when analyses of segregation kinetics indicated that the losses of some multicopy plasmids failed to conform to the expectations for a random distribution. Rather, the data were deemed to be consistent with the plasmid molecules being tethered inside the cell. These studies were conducted on ICPs paving the way for speculation that the aggregation of the plasmids might be a consequence of Rep-mediated handcuffing (described earlier). Evidence that implicates membrane association as being important for the *in vivo* functions of replisomes and Rep proteins is accumulating. In fact, some Rep proteins have amino acid signatures that are typical of transmembrane proteins. It would not be unreasonable, then, to suspect that these DNA-binding, membrane-binding proteins might act as effectors of intracellular plasmid localization. Even if true, however, the mechanisms that account for this type of plasmid localization are most likely independent of the classic partitioning systems, which stabilize plasmids without affecting their copy numbers.

Addiction Modules

Like all living organisms, bacteria die, and plasmids are known to facilitate this ultimate stage of life. Bacterial addiction to plasmids is a very basic, and at first glance counterintuitive, phenomenon, making its discovery exciting. One of the best studied forms of death in bacteria is mediated through specific genetic components called 'addiction modules' or toxin–antitoxin systems. Each consists of a pair of genes, a stable toxin and an unstable antitoxin that interferes with the toxin's lethal action. The existence of these plasmid-encoded elements was inferred as a result of some interesting observations arising from studies (in *E. coli*) of low copy number plasmids such as Rts1, RI, and F. It was found that a mutant Rts1 plasmid with temperature-sensitive machinery for plasmid replication had the unexpectedly broader phenotype of making the growth of its bacterial host cells temperature sensitive as well. Dissecting the phenomenon revealed that at the nonpermissive temperature, the bacterial host cells lost all copies of Rtsl, and with it all copies of the antitoxin. Degradation of the antitoxin and its messenger RNA left the unneutralized toxin to linger in the cytoplasm and kill the plasmid's former host. The Rts1 locus that was responsible for this effect that was later referred to as segregational killing, a term arising from studies of the analogous *hok/sok* locus of plasmid RI. Over the years, a variety of different genetic systems have been described that promote either bacteriostatic or bacteriocidal effects in bacteria. In all cases, a cell that liberates itself from the forced symbiosis with the plasmid DNA will most likely die or stop growing due to the activity of long-lived toxins.

The highly unusual behavior of plasmid-bearing cells eventually led to a frontal attack on the chromosomally encoded toxin–antitoxin systems, some of which are homologous to the plasmid addiction modules. *E. coli*'s *mazEF*, for example, is a stress-induced 'suicide module' that activates when a stressful condition interrupts the expression of MazE allowing the protein to exert its toxic effect and cause cell death. The presence of *mazEF*-like modules in the chromosomes of many bacteria suggests that cell death plays roles in bacterial physiology and/or evolution. Furthermore, there are observations suggesting an interplay between the plasmid- and chromosome-encoded addiction systems. For example, the toxic product (Doc) of the *phd/doc* addiction module of the plasmid prophage P1 requires the presence of the cellular *mazEF* system to be bactericidal. Bacterial addiction modules are often classified as mechanisms of programmed cell death (PCD) or apoptosis, terms that are traditionally associated with eukaryotic multicellular organisms. Future studies of plasmid addiction and other PCD systems in bacteria will be important for revealing the death pathways involved and perhaps for designing new classes of antimicrobial agents (e.g., compounds that interfere with antitoxin expression or activity).

Why would a genetic element that is potentially toxic to the genome ever be maintained? The far-reaching impact of the discovery of plasmid addiction is that it has fostered an important conceptual change in our understanding of bacteria. Death is clearly counterproductive for an individual bacterium; however, it might be advantageous for a whole cell population. Growing experimental evidence suggests that bacteria seldom behave as individual organisms. In fact, some species have evolved the ability to communicate with each other via quorum-sensing signal molecules, which allow coordinated responses to a variety of stimuli. As a result, bacteria can be induced to manifest multicellular-like behaviors and addiction may fall into this category. Plasmid-encoded and chromosomal toxin–antitoxin systems with their attendant killing of 'afflicted' bacteria can be viewed as examples of multicellular behaviors under stressful conditions. When challenged, the bacterial population seems to act like a closed society in which a subpopulation is excluded through forced suicide, thereby permitting the survival of the bacterial population, with its genome remaining intact.

Horizontal Plasmid Transfer by Conjugation

Bacteria can acquire foreign DNA by various means including phage-mediated delivery (transduction) and the uptake of 'naked' DNA (transformation). In addition, certain plasmids are equipped to facilitate lateral gene transfer between bacteria through a process, mentioned earlier in this article. Conjugation is usually mediated by plasmids and transposons, and important details differ from system to system as a consequence of plasmid diversity (Conjugation, bacterial and transposable elements). Conjugative plasmids rely, at least in part, on plasmid-borne gene products and specific DNA sequences to transfer themselves from hosts to recipient cells. One of the more simple conjugation systems, as judged by the number of participants it employs, can be found in mycelial streptomycetes. A single plasmid-encoded protein, the DNA translocator TraB, is sufficient to promote conjugal transfer of DNA in these organisms. Following primary transfer from the donor into the recipient, the plasmid is further distributed to the neighboring mycelial compartments in a process that requires 5–6 host-encoded proteins. In contrast, plasmid-encoded factors play a much more prominent role in the conjugation processes of Gram-negative and Gram-positive bacteria, and they include DNA sequences such as *oriT* in addition to multiple conjugation-related proteins. Most conjugative plasmids depend on a relaxase to start the DNA-processing reactions, streptomyces being the remarkable exception to this rule.

Coupling Mechanisms for Donor–Recipient Pairs

Bacterial conjugative systems are grouped together into the type IV secretion system (TIVSS) and the proteins required for conjugal transfer fall into three groups. Mobilization proteins (Mob and nickase, frequently called the relaxosome) bind specifically to their cognate *oriT*s and produce a nick in the DNA from which the conjugal transfer begins, utilizing the RC-like pathway described earlier in the article. Transfer proteins (Tra) form a multiprotein complex called the mating pair formation apparatus (Mpf) that, among other transport-related activities, is needed for pili formation and their extrusion to the cell surface. Tra proteins are not functionally confined to the *oriT* of the same plasmid. They can facilitate the transfer of plasmids that contain unrelated *oriT*s provided those plasmids also contain the cognate relaxosomes. Once plasmid DNA is prepared for transfer, it must be transported through the donor's cytoplasmic membrane into the recipient. It is generally believed that DNA crosses the donor membrane with the aid of a coupling protein, so-called because it couples T-strand DNA processing to a TIVSS. Free-living (planktonic) as well as surface-bound bacteria are capable of transferring plasmids, and one of the few diversifying elements in conjugation systems is the type of pili produced and used to facilitate conjugation; some pili are thick and flexible while others are long and rigid. Pili have the remarkable ability to retract, allowing them to promote intimate associations of cell surfaces over extended areas, which stabilize mating pairs against shearing forces. Plasmids signal their occupancy of the cell through a mechanism called surface exclusion that prompts bacterial cells to disengage before a redundant transmission of DNA occurs. Abundant membrane proteins called exclusion proteins are presented on the cell surface, mediating this process.

Well-studied Gram-positive conjugation systems must employ a different strategy to bring plasmid donors in contact with potential recipients since no pili have been functionally linked to conjugation in this group of bacteria. Rather, small molecules called pheromones are known (e.g., pCF10) or suspected (e.g., pAW63) to facilitate cell-to-cell contact. Pheromones are peptides of seven or eight amino acids and are secreted in miniscule amounts, with as little as 1–10 molecules per donor needed to initiate the mating process. A given pheromone specifically activates the conjugative transfer system of a particular plasmid type. When a plasmid is acquired, secretion of the related pheromone is prevented, while unrelated pheromones continue to be produced to 'seduce' other potential donor cells. The capacity of several plasmids to produce a surface-exposed aggregation protein (*in vitro* and *in vivo*) in response to pheromones expressed during conjugation has been well established and, in the case of *Enterococcus faecalis*, functionally connected to the pathology of the plasmid-bearing organism.

Factors Affecting Plasmid-Mediated Horizontal Gene Transfer

In general, conjugation-mediated transfer of DNA appears to have few if any barriers. It can occur between Gram-negative and Gram-positive bacteria as well as fungi, actinomycetes, and the cells of higher eukaryotes. Some plasmids can be remarkably promiscuous as exemplified by two antibiotic-resistance plasmids: RK2 (also known as RP4), which was originally identified in a Gram-negative host, and pIP501, which was isolated from a Gram-positive strain. pIP501 has an extremely broad host range (BHR) for conjugative transfer that includes a variety of Gram-positive bacteria, multicellular *Streptomyces*, and the Gram-negative *E. coli*. However, as impressive as this list is, RK2's conjugative spectrum is even broader as it can be transferred to all tested Gram-negative bacteria, yeast, and even mammalian cells (Horizontal gene transfer: Uptake of extracellular DNA by bacteria).

Conjugation efficiencies vary from one plasmid system to another and are affected by a plethora of factors. For many plasmids, conjugative functions are typically found to be in a repressed state and conjugation efficiency is very low. One out of million plasmid-free cells, or even less, may receive a conjugative plasmid even when the donor cells greatly outnumber the potential recipients. Interestingly, in repressed systems, transfer-proficient donors and potential recipients can initiate a cascade of conjugative transfer because the newly transferred plasmids are transiently derepressed in response to the initial lack of repressor proteins in the recipient. Mutations in repressor genes can also cause conjugation to become derepressed (*drd* mutants). In fact, in a couple of well-studied plasmids, transfer functions are naturally derepressed by deletion or insertion mutations in the gene encoding the repressor. Remarkably, these derepressed plasmid systems are able to sustain conjugative DNA transfer under laboratory conditions with close to 100% efficiency (e.g., F and pCF10). Conjugation typically occurs within aggregates of multiple donor and recipient cells; in some cases twenty and in other cases thousands of aggregated and conjugating cells were observed.

Consecutive DNA transfer events by the same donor are known to occur rapidly with estimated cycles of as little as 3–5 min for some pheromone-responsive and pili-mediated machineries (e.g., pCF10 and F). The DNA transfer process occurs at the speed of replication previously noted, roughly 1000 bp/s. Interestingly, cells that acquired plasmid DNA (newly formed transconjugants) require much longer periods, up to about 60 min, to mature into proficient donors. This long maturation time is most likely required to synthesize and assemble the impressive protein machineries used in the multiple coordinated processes that contribute to conjugation: assembling and anchoring the relaxosome (into the cytoplasmic membrane) and the Mfp components (into the cell wall), then connecting both by a coupling protein. Once assembled, the DNA secretion machinery can be remarkably stable, and cells killed by several bactericidal treatments retain conjugation proficiency for several hours. In a related and unsettling finding, externally added antibiotics (e.g., tetracycline) can stimulate some conjugative systems up to 100-fold and this derepression may not be limited to laboratory settings. These observations may necessitate changes in the way we think about horizontal gene transfer (HGT) and its potential impact on microbial communities. We will return to this topic later in the article.

Environmental cues

The acquisition of plasmids via conjugative transmission has been studied in numerous environments, providing ample evidence that both abiotic and biotic cues affect this process. Nonetheless, the focus of pioneering studies in this field needs to be narrower than ‘the environment’. Studies of the environmental factors that affect plasmid transfer rely on the use of discrete habitat subsamples called microcosms. Examples of microcosms include soils, plants, and water, all of which are being used to elucidate the effects of key ecological factors on the plasmid transfer process. Interested readers are advised to consult the Further Reading section for broader access to existing knowledge in this important area.

Spatially structured microbial populations known as biofilms can form in the microcosms described as well as in clinical systems, and they appear to represent unifying experimental systems for studying plasmid transfer processes. To dissect the conjugation process in ‘natural’ and artificial (laboratory) microcosms, an experimental approach for the direct *in situ* monitoring of plasmid transfer in biofilms has been developed. Using plasmid-encoded green fluorescent protein as a visual ‘reporter’, the intercellular movement of plasmids can be studied microscopically. Researchers have used this technique to demonstrate that conjugation has a dramatic stimulatory effect on the ability of transconjugant bacteria to participate in biofilm formation. The uptake of conjugative plasmids not only enhances the adhesive properties of bacteria but in some situations it can also dramatically restrict cellular motility, changes that are likely beneficial for surface-dwelling populations and of little to no value for planktonic cells. It would appear that biofilm formation and conjugation might be mutually reinforcing phenomena. Relevant to this, biofilms are sometimes regarded as ideal niches for conjugation although the relatively early stage of this work prevents general conclusions from being drawn. New mathematical approaches that model the spatial dynamics of plasmid transfer and persistence are increasingly being turned to, their purpose being to ascertain how three-dimensional structure affects the spread and loss of plasmids in surface-associated bacterial communities. Plasmid ecology is also studied in biofilm microcosms with the long-term objective of breaking down toxic compounds in wastewaters and other environments by disseminating degradative genes via conjugation. ‘Three-dimensional’ images of conjugating biofilm communities have been generated and transconjugants (tagged with green fluorescent protein) in different locations within the biofilm were observed. Scientists perform studies like these with hope of predicting how HGT might work in practical applications of conjugation in natural environmental contexts such as bioremediation and other emerging applications.

Restriction enzymes

One of the potential obstacles confronting the conjugal transfer of plasmid DNA is a group of host-encoded proteins, the restriction enzymes (RE), that are designed by nature to destroy DNA they recognize (DNA restriction and modification). Their resemblance to the addiction cassettes described earlier is evident in that they work with companion enzymes of opposing function, one enzyme cleaves DNA (analogous to toxin) and the other modifies the DNA recognition sequence thereby preventing DNA damage (analogous to antitoxin). Such restriction–modification (RM) systems protect cells from an invasion by foreign DNA. They are ubiquitous in bacteria and can be plasmid-encoded or reside on the bacterial chromosome. Although the DNA that is transferred during conjugation is single-strand and therefore not susceptible to restriction, there is a race to protect or restrict as the T-strand is converted into double-strand DNA.

There is little doubt that RM systems affect the efficiency of plasmid spread. Conjugation and plasmid establishment are expected to occur more frequently between taxonomically related species in which plasmid DNA can evade restriction systems and replicate. It comes as no surprise, therefore, that the DNA of some BHR plasmids, which are capable of replicating in many hosts, contains fewer restriction sites when compared to the DNA of their narrow host range counterparts.

Additionally, many conjugative plasmids contain anti-restriction loci (*ard*) as part of their so-called 'leading region' defined as the first portion of the plasmid to enter the recipient. The products of these genes act specifically to alleviate restriction by certain types of RE. Having an *ardA* locus present in *cis* allows an incoming, unmodified plasmid to evade restriction when transferred by conjugation but not by other processes that involve double-strand DNA (e.g., transformation or electroporation). Protection requires the expression of the incoming *ard* gene, which is enhanced by conjugative transport into the recipient cell.

Replication Ranges of Conjugative Plasmids

The conjugation ranges of plasmids and their replication ranges are related but distinct entities. The replication range refers to the variety of hosts that can maintain an extrachromosomal plasmid once it enters a cell; this range is typically narrower than the conjugation range. Despite the higher demands posed by plasmid maintenance, however, BHR plasmids are able to replicate in a diverse assortment of bacteria, employing various strategies to achieve their promiscuity. One strategy adopted by some BHR plasmids is to limit their reliance on host proteins by encoding their own helicase and primase. As a result, these plasmids have an advantage, but successful maintenance in any given strain is not assured. BHR replisome assemblies still require the expression of plasmid-encoded proteins in the bacterial host, productive interactions between the plasmid and accessory host proteins, and productive interactions between the host proteins and DNA-binding sites of the plasmid. Moreover, plasmid-encoded proteins must be expressed at an appropriate level, possibly even at a specific time in the cell cycle, and the proteins must be stable in different host backgrounds.

Plasmids of the IncP group forgo encoding their own replisome proteins and employ two alternate strategies that enhance their replicative promiscuity. First, they produce a Rep that is versatile enough to recruit helicases of distantly related bacterial species. Indeed, *in vitro* and *in vivo* work have demonstrated that plasmids of this group use different pathways for helicase recruitment and activation. Structural differences in DnaB helicases from different species of bacteria are likely the basis for the diversity required to form a productive interaction. The second mechanism is more elaborate and reflects a unique interrelationship between DNA transfer and vegetative replication modes. Specifically, IncP conjugation systems have the unusual ability to transfer (unidirectionally) primases and single-strand DNA-binding proteins (SSB) into the recipient bacterium as nucleoprotein complexes. Transfer is abundant, amounting to several hundred molecules of primase. The enzyme contributes to transfer promiscuity by eliminating the requirement for the host enzymes of different bacteria to recognize the incoming DNA strand, which facilitates efficient second-strand synthesis in different cellular backgrounds. SSB proteins, which are also transferred via the conjugation apparatus, are essential for DNA replication and repair. The traveling SSBs encoded by BHR plasmids presumably overcome deficiencies of host SSB in cells receiving single-strand DNA during conjugation, presumably leaving the metabolism of chromosomal DNA (replication and recombination) relatively undisturbed.

Although naturally occurring plasmids with multiple *ori*s have been isolated (e.g., R6K), none has been shown to specifically utilize different *ori*s in different bacterial hosts. Nonetheless, engineered plasmids called 'shuttle vectors' contain two distinct replicons that are active in unrelated hosts and they prove that the presence of two narrow host range replicons on a single plasmid can extend its host range. Narrow host ranges can also be broadened by mutations in genes that encode an essential plasmid or host protein, the consequence of which is likely the strengthening of a required host–protein/plasmid–protein interaction (e.g., pPSlO and DnaA/RepA).

In summary, the ability of a plasmid to transfer itself from one bacterium to another by conjugation or to be mobilized between hosts by conjugative functions provides the means by which a plasmid can pioneer new cellular landscapes. Once an immigrant plasmid is introduced, many pieces must come into play in a well-orchestrated manner for a plasmid to be able to survive. While restriction and replication processes are key, other factors contribute to plasmid promiscuity. Analyses of the partitioning and postsegregational killing systems clearly demonstrate the role of accessory functions in extending or limiting plasmids' ability to be maintained. To be of use, a variety of contributing elements must be expressed and regulated in novel hosts. It is evident that we are just beginning to understand some of the key genetic and molecular factors involved in extending the host range of plasmids in all three kingdoms of life.

Plasmid Evolution

Evolutionary analysis relies upon the identification of unifying features. The last three decades have seen tremendous advances in the determination of the evolutionary relationships that connect all living organisms. The use of 16S ribosomal RNA genes to determine phylogenetic relationships has provided a unifying methodology for evolutionary analysis even as it resulted in recognizing a new branch in the tree of life. That tree is divided into the three domains (bacteria, archaea, and eukarya), and plasmids inhabit organisms belonging to all three domains but certainly are most prevalent in bacteria. Plasmids are not organisms in their own right; instead, they represent a horizontal gene pool, which is coevolving with their hosts. Not surprisingly, a signature DNA sequence such as 16S ribosomal RNA is lacking in plasmids primarily because the very nature of these elements is to not encode essential host information.

Determining the evolutionary relatedness of replicons

Even the fundamental ability to replicate cannot be used to establish the common ancestor to all plasmids, as evidenced by the inherent diversity of replicons. It appears that plasmid replication functions likely originated more than once, independently of each other. However, the absence of a single identifiable reference sequence in all plasmid genomes has not impeded the construction of adequate phylogenies encompassing groups of plasmids. In fact, the debate about plasmid classification is reminiscent of the ongoing discussion regarding the concept of bacterial species. What level of DNA similarity makes it reasonable for researchers to contend that plasmids belong to the same plasmid group? Another issue is to decide what genes/sequences are best suited to generate plasmid phylogenies. The main contenders are the genes coding for the Rep (**Figure 13**) and Par proteins and the proteins facilitating DNA transfer. Homologies in these groups of proteins and their cognate DNA targets have been established and are emphasized on numerous occasions in the literature pertaining to this important topic. An example phylogeny based on the *rep* gene sequences of theta-type replicons is shown in **Figure 13**. However, as will be discussed, these phylogenies are valuable only in establishing the evolutionary relationship of plasmid 'genes'; the task of establishing

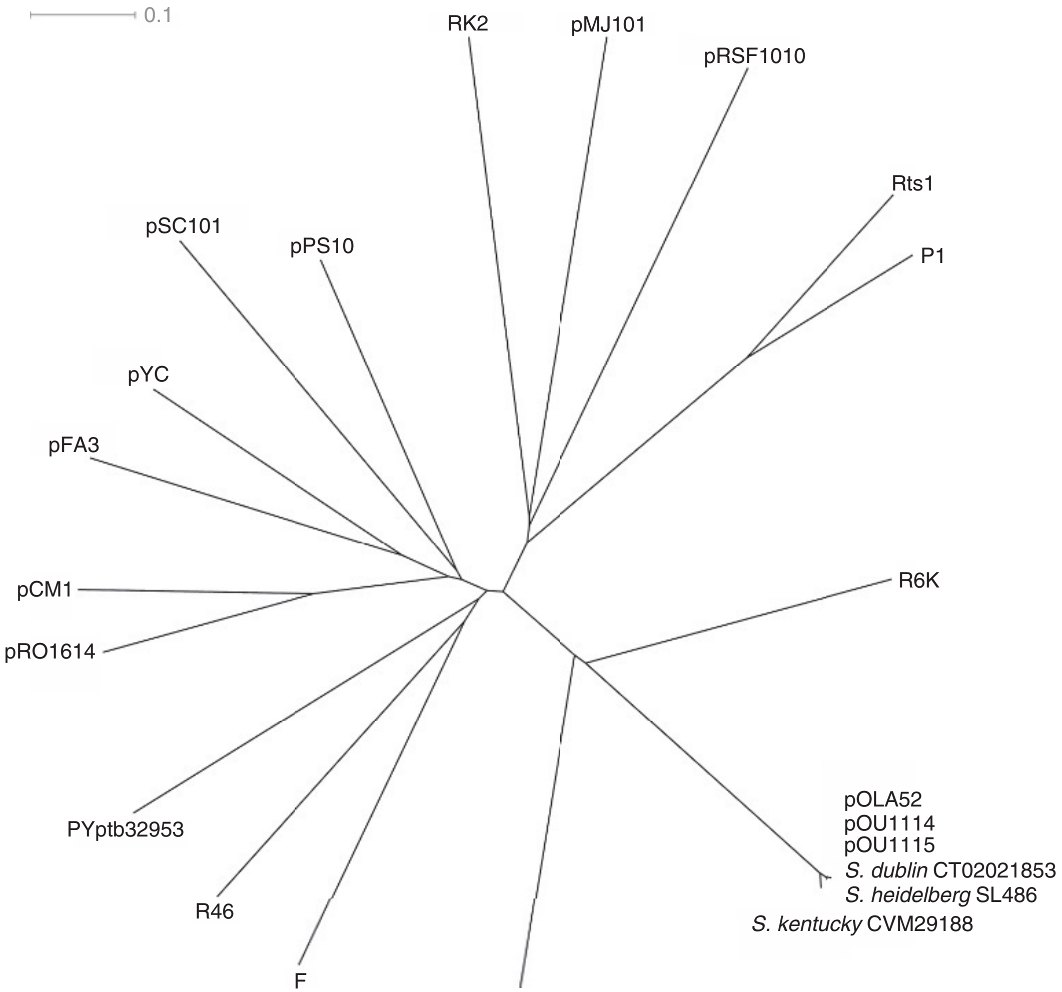

Figure 13 Phylogenetic tree based on alignments of Rep proteins of various iteron-controlled plasmids or genomic sequences (strain names in italic). Alignments were made with Clustal W and the tree was constructed using SplitsTree 4.8. Protein sequences were obtained from GenBank either directly or by translating nucleotide sequences of putative genes located with Glimmer 3.02. Reprinted from Norman A, Hansen L H, She Q, and Sørensen (2008) Nucleotide sequence of *pOLA52*: A conjugative *Inc*X1 plasmid from *Escherichia coli* which enables biofilm formation and multidrug efflux. *Plasmid* 60: 59–74, with permission from Elsevier.

relationships between plasmids in their entirety is substantially more complex. Included in the Further Reading are references to the sources of plasmid DNA sequences. Resources such as these have been multiplying rapidly since the inception of research programs specifically designed for sequencing plasmids and annotating their genes (e.g., Wellcome Trust SANGER Institute).

There is no doubt that plasmid classification systems based on DNA sequence similarities in the segments that make up the plasmid 'backbone' (replication, maintenance, and transfer regions) is gaining more and more importance. The increasing number of sequenced plasmids has prompted the use of DNA primers to amplify and determine the DNA sequence of plasmid backbone-specific segments, which in theory should help establish the relatedness of 'new' plasmids to 'known' ones. Attempts have been made to sample new plasmids from a variety of exotic and mundane environments using sequencing primers that recognize select, known plasmids from Enterobacteriaceae. However, these attempts have overwhelmingly failed to detect any relatedness in these isolates to the well-characterized *ori*s. Clearly, much more remains to be discovered in our ongoing quest to sequence the mass of collective environmental DNA samples (i.e., metagenomes, discussed in Metagenomics). How many more signatures of new replicons will we find? How much will we learn about where they originated and for what 'purpose'? So far, science has only touched the tip of an iceberg when it comes to uncovering the diversity of replication *ori*s and their attendant genes for both chromosomal and plasmid origins.

Plasmids as vehicles of genetic plasticity

Since the discovery of plasmids, we have learned that besides the machinery for their own maintenance and transfer, most also carry genes that confer a plethora of traits on their bacterial hosts. Frequently, these traits are ones that are useful intermittently or in certain environments, such as antibiotic resistance, virulence, or degradation of unusual substrates (some discussed below). The various functions, found on both circular or linear plasmids isolated from nature, can be as simple as a single gene (e.g., an antibiotic-resistance determinant) or may involve genes encoding whole metabolic pathways requiring hundreds of kilobases of DNA sequence (e.g., nitrogen fixation in rhizobia). Before we discuss some specific examples, it should be stressed that the factors that contribute to evolutionary change in plasmids are the same as those which are involved in evolution in general – single base pair substitutions, insertions, deletions, and genetic rearrangements such as inversions and translocations. The high adaptability of plasmid-bearing strains relies on various recombination events, which may occur at the borders of functional units with 'recombinogenic ends' designed for recombination (e.g., transposons and integrons; Transposable elements) or as a result of HR, often between parental and newly synthesized DNA (described earlier and Recombination, genetic). In fact, recent investigations have suggested that recombination between genes in plasmids may occur at a much higher frequency than chromosomal recombination. The mechanism accounting for this apparent difference remains to be determined.

Hundreds of plasmid and bacterial genome sequences already available have revealed extensive HGT within and between these classes of replicons (DNA sequencing and Genomics, Genome Sequence Databases: Annotation, and Horizontal gene transfer: Uptake of extracellular DNA by bacteria). On an evolutionary scale, plasmid-mediated gene rearrangements appear to be particularly significant. Science continues to discover that genomes are full of mobile genetic elements and there is compelling evidence that many genes have joined bacterial genomes relatively recently from distantly related organisms, even from eukaryotes. In addition, comparisons of closely related genome sequences (e.g., *E. coli* K12 and *E. coli O157:H7*) suggest frequent rearrangements of DNA during their evolution. Perhaps related to this, most circular plasmids contain site-specific recombinase genes and the multiplicity of these genes found on large plasmids often correlates with their level of mosaicism. In other words, the greater the number of recombinases, the more likely it is that a plasmid will be regarded as a mishmash of sequences derived from multiple sources. Evidence exists that 'illegitimate' recombination mediated by plasmid and transposon-encoded resolvases (i.e., DNA inversion and intermolecular fusion reactions) has also contributed to plasmid evolution. Indeed, it has become apparent that plasmids are quite active in shuffling, recombining, and redistributing genes or sets of genes, and in so doing they facilitate not only their own evolution but also the evolution of microbial communities and individual strains.

Antibiotic resistance: An example of plasmid-enhanced bacterial adaptability

The classic principle of genetic selection of fitness is painfully illustrated by the fact that conventional antibiotic treatments are becoming increasingly ineffective due to the acquisition and dissemination of antibiotic-resistance genes by bacteria. In fact, the continuous manifestation of an antibiotic-resistance phenomenon is not new. The first observation of resistance to penicillin was described before the drug was even in clinical use. Furthermore, it was already evident in the 1950s, from the work of plasmid researchers in Japan, that antibiotic resistance was on the rise – and it has been increasing dramatically ever since. There are many examples of the astonishingly rapid acquisition of antibiotic resistance by bacteria. One particularly remarkable and disturbing example is found in the opportunistic pathogen *Acinetobacter baumannii*, which has acquired close to 50 resistance genes in just 40 years! Integrons and associated gene cassettes (**Figure 14**) have been shown to be of major importance in the acquisition of antibiotic-resistance genes by this and other species.

Several factors have played a pivotal role in the remarkable speed with which bacteria have adapted to our chemical arsenal. First of all, antibiotics do not specifically target bacteria that cause infections; they are indiscriminate killers of susceptible bacteria – be they harmful, benign, or beneficial organisms. This becomes problematic on the recognition that resistance genes are rarely fixed in the chromosome of a bacterial cell, rarely restricted in their transmission to only that cell's progeny. Instead, such genes are typically found on transmissible plasmids and transposons. The transmission problem becomes further exacerbated in specific cases where antibiotics can stimulate transposition such as those that occur in the human commensal organism, *Bacteroides*. Another possible factor in the rapid dissemination of resistance, noted earlier in the article, is the ability of bacteria harboring plasmids to continue transferring the DNA to other cells long after the donating bacterium has been killed. Finally, cells under various forms of stress have higher mutation rates. As a result, antibiotics that cause DNA damage, such as mitomycin C, can directly elevate the frequency of mutation. Remarkably, antibiotics that affect translation fidelity also boost the mutation rate in bacteria.

All the factors just described along with others that are probably yet to be discovered allow reservoirs of resistance to emerge and rapidly spread within diverse microbial communities (**Figure 14**). Although niches

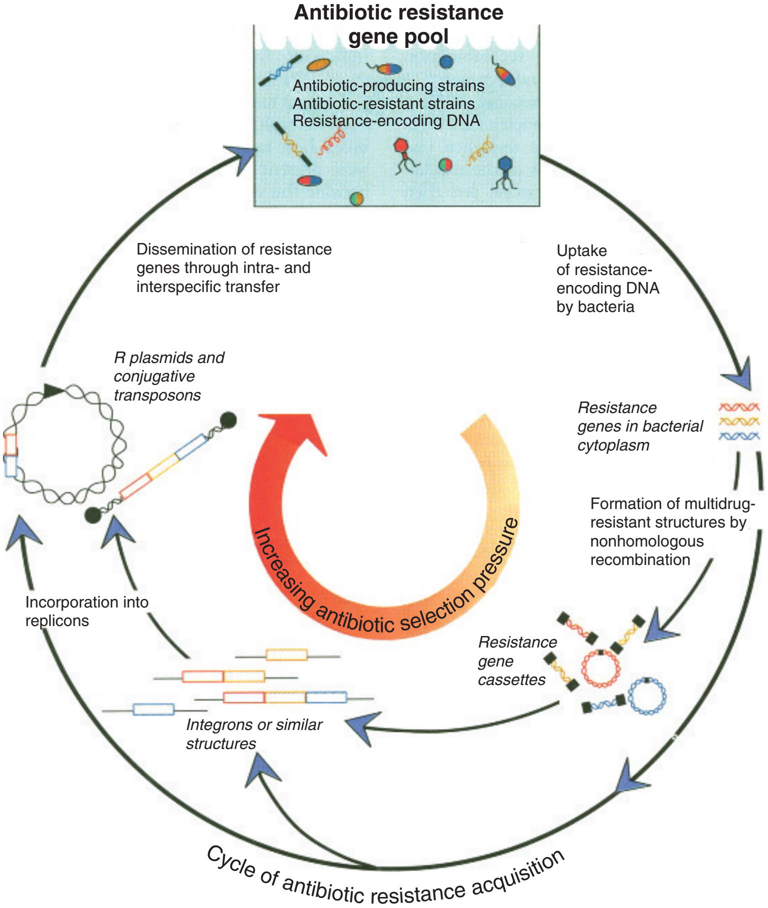
Antibiotic resistance gene pool
Antibiotic-producing strains
Antibiotic-resistant strains
Resistance-encoding DNA
Uptake of resistance-encoding DNA by bacteria
Resistance genes in bacterial cytoplasm
Formation of multidrug-resistant structures by nonhomologous recombination
Resistance gene cassettes
Integrons or similar structures
Incorporation into replicons
R plasmids and conjugative transposons
Dissemination of resistance genes through intra- and interspecific transfer
Increasing antibiotic selection pressure
Cycle of antibiotic resistance acquisition

Figure 14 A scheme showing the route by which antibiotic-resistance genes are acquired by bacteria in response to the selection pressure of antibiotic use. The resistance gene pool represents all potential sources of DNA encoding antibiotic-resistance determinants in the environment; this includes hospitals, farms, or other microenvironments where antibiotics are used to control bacterial development. After uptake of single- or double-stranded DNA by the bacterial host, the incorporation of the resistance genes into stable replicons (DNA elements capable of autonomous replication) may take place by different pathways, which have not yet been identified. The involvement of integrons, as shown here, has been demonstrated for a large class of transposable elements in the Enterobacteriaceae. The resulting resistance plasmids could exist in linear or circular form in bacterial hosts. The final step in the cycle, dissemination, is brought about by one or more gene transfer mechanisms discussed in the text. From Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264: 375–382. Reprinted with permission from AAAS.

such as the human or animal gut, manure, sewage, soils, plant surfaces, and water systems are often thought of as being distinct, they are actually microbiologically connected. Favorable plasmid-borne genes can be found to circulate between different microenvironments and selective pressure by antibiotics might exacerbate this genetic exchange. For instance, there is evidence identifying very similar replicons with very similar streptomycin- and tetracycline- resistance genes in diverse hosts in two distinct habitats, clinical hospitals and agricultural fruit orchards. The fact that both the 'habitats' have been subjected to streptomycin and tetracycline selective pressure for decades is unlikely to be a coincidence. From the foregoing discussion it is evident that a change in our understanding of microbial evolution is necessary to fully appreciate why antibiotics and other antimicrobial agents are destined to eventually have their utility undermined by resistance acquisition.

Broader contributions to microbial evolution

Another way of ascertaining plasmid–host coevolution is by monitoring novel metabolic capacities harbored by plasmid-bearing bacteria. Of particular interest, bacterial responses to toxic compounds (e.g., xenobiotics) in the natural environment have provided an opportunity to study the evolution and acquisition of new catabolic processes. Such toxins are sometimes organic in composition and include pesticides, herbicides, refrigerants, and solvents – one of which, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) – has been in use for over 50 years. Genes that break down 2,4-D are carried on a conjugal plasmid called pJP4. This plasmid has provided a model for studies of the evolution and spread of catabolic pathways in bacterial communities, a process that is mechanistically more demanding than the acquisition and spread of antibiotic-resistance genes described above. Abundant data suggest that there has been extensive interspecies transfer of pJP4. Moreover, there is evidence to suggest that the genes in the 2,4-D degradative pathway may have evolved elsewhere, for other purposes, and then recent recombinations and modifications were selected in response to 2,4-D in the environment. The adaptive transfer and reorganization of genetic modules is also well illustrated by the analysis of bacteria from polluted environments; these organisms sometimes acquire the ability to degrade chemicals that would otherwise persist for long periods of time. In this case too, the incorporation of new genetic material has been the most important mechanism for expanding metabolic pathways. The aforementioned examples of plasmid-mediated host adaptation illuminate a principle of broad significance at the interface of plasmid biology and microbial sciences: the survival of plasmids appears to be heightened by genes that provide selective advantages to their host organisms. In that light, perhaps 'selfish' is a little harsh as a descriptor of these versatile and diverse conduits of bacterial evolution. But admittedly, 'benevolently self-interested DNA' really does not have the same cachet.

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http://www.sanger.ac.uk – The Wellcome Trust Sanger Institute
http://www.essex.ac.uk – University of Essex

Posttranscriptional Regulation

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Defining Statement
Introduction
Regulation of Premature Termination of Transcription
Regulation of mRNA Stability
Regulation of Translation
Conclusion
Further Reading

Glossary

antiterminator RNA element that prevents the activity of an attenuator.

attenuator Terminator located in the leader region of a transcriptional unit, used to regulate expression of downstream genes.

autogenous regulation Regulation of a gene by the product encoded by that gene.

cis-*acting element* Element that affects only a region with which it is physically connected (e.g., a DNA site).

coupling Coordination of cellular processes, for example, transcription and translation.

leader peptide Short peptide encoded in the leader region of a transcriptional unit.

leader region Segment of a gene between the transcription initiation site and the start of the coding sequence.

leader RNA Segment of an RNA that is upstream of the first coding sequence.

ribosome binding site (RBS) Translation initiation signal, comprised of SD plus start codon (usually AUG, GUG, or UUG).

Shine–Dalgarno sequence (SD) Binding site for the 30S ribosome on an mRNA for translation initiation.

terminator Signal for RNA polymerase to stop transcription and release the DNA template and the nascent transcript.

trans-*acting factor* Factor or substance that can act at a distance within a cell (e.g., a protein or RNA that can diffuse to a different site).

transcription elongation complex RNAP as it moves along the DNA template, after leaving the promoter site.

Abbreviations

5′ UTR	5′ untranslated region
PNPase (I occurrence)	polynucleotide phosphorylase
RBS	ribosome binding site
RF2	release factor 2
RNAP	RNA polymerase
SAM	*S*-adenosylmethionine
SD	Shine–Dalgarno sequence
sRNAs	small regulatory RNAs
$\mathbf{tRNA^{Trp}}$	tryptophanyl-tRNA

Defining Statement

Posttranscriptional gene expression events that occur after RNA polymerase (RNAP) leaves its promoter site offer many opportunities for regulation. This article reviews some of these mechanisms, including modulation of premature termination of transcription, mRNA degradation, and translation initiation, and also illustrates each class with specific examples.

Introduction

Regulation of gene expression at the level of transcription initiation is of obvious importance in all biological systems. Recognition of the promoter site on the DNA by RNA polymerase (RNAP), melting of the DNA, synthesis of the first few nucleotides of the transcript, and escape from the promoter site all represent important steps at which transcription initiation can be controlled. However, it has become increasingly obvious that events that occur after RNAP leaves its promoter site offer many additional opportunities for regulation, and mechanisms that affect these events are considered to be posttranscriptional regulatory events, although certain of these mechanisms affect transcript levels. Premature termination of transcription results in failure to synthesize the complete transcript. Modulation of transcript degradation, which can begin before the transcript has been fully synthesized, can have a major effect on expression of the encoded genes. The efficiency with which an mRNA is translated can dramatically affect the synthesis of the encoded protein product and can also affect the rate of transcript degradation. This article reviews mechanisms of gene regulation that occur after transcription initiation. Specific examples are described to illustrate each class of event.

Regulation of Premature Termination of Transcription

One of the most common mechanisms of posttranscriptional gene regulation involves positioning of a transcription termination signal (or terminator) in the region between the promoter and the start of the regulated coding sequence. This region is commonly called the leader region in bacteria, and corresponds to the 5′ untranslated region (5′ UTR) in eukaryotic cells; because this region can be translated in bacteria to yield a leader peptide, as described below, leader region is a more general term. Termination signals located within leader regions are called attenuators, as their role is to attenuate synthesis of the full-length transcript. Modulation of the activity of the terminator element in response to an appropriate physiological signal can therefore be used to determine the amount of full-length transcript made and the level of expression of the gene(s) that are downstream of the attenuation site. A wide variety of regulatory mechanisms of this type have been uncovered. Many of these mechanisms depend on effects on the folding of the nascent RNA transcript, while others affect the ability of the transcriptional machinery to recognize termination signals.

Termination Mechanisms

Two types of transcriptional terminators have been described in bacteria. Detailed biochemical analyses have been carried out primarily in *Escherichia coli*, but it appears that the basic features of terminators, and therefore their utilization as a regulatory target, are conserved in many groups of bacteria. Both types of termination events require features of the nascent RNA transcript that emerges from the elongating RNAP that the terminator is attempting to halt. The most common class of terminator elements are called intrinsic (or factor-independent) terminators. These termination signals are defined biochemically by their activity in a purified *in vitro* transcription system, in the absence of any cellular factors other than RNAP. Intrinsic terminators are comprised of a G + C-rich RNA helix, usually 7–8 nt in length, immediately followed by a stretch of U residues in the nascent transcript. Positioning of RNAP so that the U residues form the RNA–DNA hybrid within the enzyme, and the RNA helix abuts the distal portion of the enzyme, results in destabilization of the transcription complex and termination. The role of the helix has been proposed to involve pushing forward on RNAP to remove the 3′ end of the transcript from the active site of the enzyme, while the U–A RNA–DNA hybrid is likely to assist in complex destabilization. Regulation of the activity of intrinsic terminators often occurs by alternate folding of the nascent RNA transcript, so that the region necessary for formation of the 5′ side of the terminator helix is instead sequestered in an alternate structure, termed an antiterminator. Intrinsic terminators can also be affected by increasing the processivity of RNAP, which reduces the sensitivity of the transcription complex to destabilization, at least in part because RNAP passes through the termination site before the RNA helix can exert its destabilizing effect.

The second class of bacterial termination signals are designated Rho-dependent (or factor-dependent) terminators, based on the requirement for an additional protein (Rho factor) for termination activity *in vitro*. Rho binds to the nascent RNA transcript, moves along the RNA until it encounters RNAP (usually at a pause site), and promotes destabilization of the transcription complex, probably by removal of the 3′ end of the transcript from the active site of the enzyme. Binding sites for Rho on the RNA, designated *rut* (Rho utilization) sites, are poorly conserved, but are generally unstructured, with overrepresentation of C and U residues and underrepresentation of G residues. Regulation of Rho-dependent termination occurs either by sequestration of the *rut* site on the RNA to prevent Rho binding or by modification of the transcription complex to increase processivity of RNAP, interfering with the ability of Rho to reach the transcription elongation complex.

Regulation of Termination by Modulation of Nascent Transcript Structure

As noted above, the activity of intrinsic terminators is absolutely dependent on folding of the nascent transcript as it emerges from the RNA exit channel of RNAP into a helix that plays a crucial role in destabilization of the transcription elongation complex. Formation of the helix therefore represents an effective target for regulation of termination activity. A variety of factors can interact with the nascent RNA to determine whether it folds into the terminator helix, in most cases by determining whether the terminator helix or a competing antiterminator helix is formed. The relative stabilities of the terminator and antiterminator helices play a major role in determining the sensitivity of the RNA to the interacting factors, and whether binding of the factor results in attenuation of transcription or increased synthesis of the full-length transcript. Interacting factors that have been demonstrated to affect leader region terminator activities include translating ribosomes, RNA-binding proteins, *trans*-acting RNAs, and small molecules.

Leader peptide attenuation systems

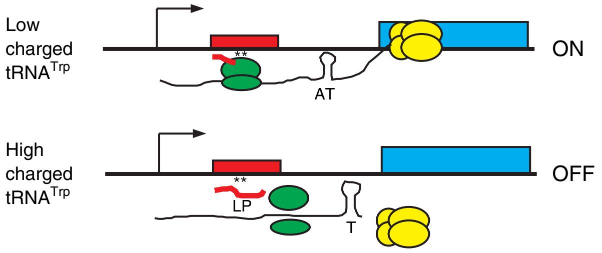
The classic model for regulation of nascent transcript structure to control termination involves the presence of a short peptide coding sequence within the 5′ region of the transcript. The processivity of the ribosome during translation of this peptide coding sequence modulates the structure of the RNA, therefore affecting both pausing of the transcription elongation complex from which this nascent RNA is emerging, and the folding of this transcript into competing terminator and antiterminator elements. Processivity of the ribosome can in turn be affected by the sequence of the nascent peptide itself. For example, in systems like the *E. coli trp* operon, which encodes gene products involved in tryptophan biosynthesis, the peptide coding sequence includes tandem tryptophan codons (**Figure 1(a)**). The presence of these tryptophan codons renders translation of the leader peptide coding region sensitive to the availability of charged tryptophanyl-tRNA (tRNA^{Trp}). Reduced abundance of tryptophan results in a reduction of charged tRNA^{Trp}, causing the translating ribosome to stall as it attempts to translate the tandem tryptophan codons. The stalled ribosome allows the unoccupied RNA ahead of it to fold into an antiterminator element, which prevents formation of the more stable terminator helix, therefore resulting in readthrough of the leader region transcription terminator site (or attenuator), and synthesis of the full-length transcript. The presence of a large pool of charged tRNA^{Trp} results in efficient translation of the leader peptide coding sequence. Rapid progress of the ribosome through the leader peptide coding region prevents formation of the antiterminator, allowing formation of the terminator helix and attenuation of transcription. Synthesis of the full-length transcript, and therefore expression of the tryptophan biosynthesis genes, therefore occurs only when cells are limited for charged tRNA^{Trp}, which signals a requirement for increased tryptophan biosynthetic activity. This type of mechanism is easily converted to allow recognition of a different charged tRNA by changing the sequence of the leader peptide itself, so that the tryptophan codons in the nascent RNA are replaced with clusters of codons specifying a new amino acid (e.g., the leader peptide of the histidine biosynthetic operon contains multiple histidine codons).

This type of transcription termination control mechanism is often superimposed on a second level of regulation. For example, initiation of transcription of the *E. coli trp* operon is regulated by the TrpR DNA-binding protein, which blocks binding of RNAP to the promoter region and represses transcription initiation when tryptophan levels are high. The combination of the two regulatory mechanisms allows sensing of both free tryptophan (by TrpR) and charged tRNA^{Trp} (by the ribosome that is translating the leader peptide coding sequence). This results in a highly sensitive regulatory response that prevents wasteful expression of *trp* operon genes when tryptophan is abundant and also ensures an adequate supply of charged tRNA^{Trp} for efficient protein synthesis.

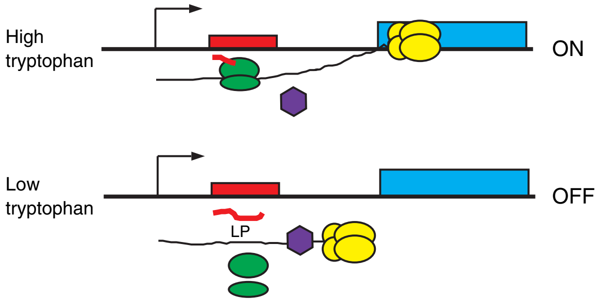
Leader peptide transcription attenuation mechanisms are dependent on the tight coupling of transcription and translation in bacteria, where binding of the 30S ribosomal subunit to an RNA transcript can occur as soon as the RBS emerges from the transcription elongation complex. Translation of the leader peptide coding sequence must be coordinated with pausing of RNAP during synthesis of the leader region of the transcript to allow a response to ribosome positioning before RNAP escapes from the attenuator region or terminates transcription. In the *E. coli trp* system, a specific signal in the leader RNA causes RNAP to pause, allowing translation of the leader peptide to initiate; the translating ribosome releases RNAP from the pause site, so that transcription and translation now proceed in tandem unless the ribosome is stalled by low abundance of charged tRNA^{Trp}.

Coupling of transcription and translation is also crucial to the *E. coli pyrB1* attenuation mechanism. This operon encodes products involved in biosynthesis of pyrimidine nucleotides, and direct sensing of NTP abundance by the transcription elongation complex is used to repress expression when pools of these nucleotides are high. Pausing of RNAP during transcription of segments of the leader region containing runs of C and U residues is triggered by limitation for pyrimidine nucleotides. This pause allows time for initiation of translation of a leader peptide coding sequence, and positioning of the translating ribosome on the nascent RNA prevents formation of the terminator helix. When pyrimidine nucleotides are abundant, rapid progress of RNAP through the pause sites before the ribosome can bind and occlude the terminator allows termination, and repression of *pyr* operon expression, to occur. As in the *E. coli trp* system, the position of a translating ribosome relative to the transcribing RNAP determines the structure of the nascent RNA, and therefore regulates whether transcription is attenuated within the leader region. In the *pyrB1* system, RNAP serves as the molecular sensor for the effector molecule (pyrimidine nucleotides),

(a) Transcriptional attenuation: *E. coli trp*



(b) Rho-dependent termination: *E. coli tna*



(c) Translation initiation: Gram-positive *cat*

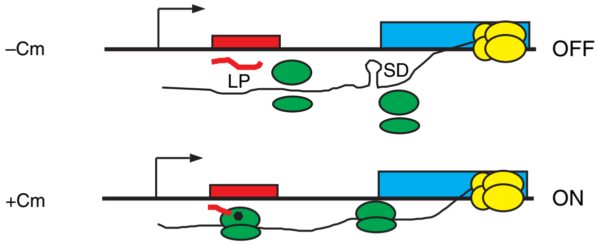


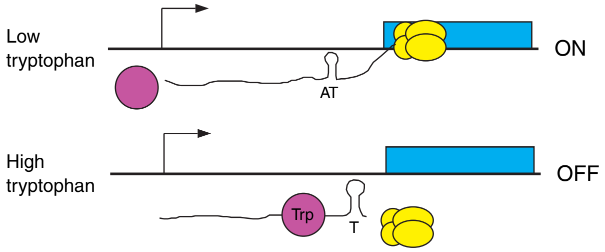
Figure 1 Leader peptide-mediated effects on gene expression. (a) Transcriptional attenuation in the *Escherichia coli trp* operon. Translation of the leader peptide coding sequence (red box), which includes two tandem tryptophan codons (**), results in stalling of the ribosome when availability of charged tRNA^{Trp} is low; this allows the nascent RNA to form into an antiterminator structure (AT) that permits RNAP (yellow ovals) to proceed through the attenuator site, and transcribe the downstream gene (blue box). High availability of charged tRNA^{Trp} causes efficient leader peptide (LP, red line) translation, and the movement of the ribosome (green ovals) causes folding of the leader RNA into the terminator helix (T); this prevents synthesis of the full-length transcript, and expression of the downstream genes is repressed. (b) Regulation of Rho-dependent transcription termination in the *E. coli tna* operon. The ribosome translating the leader peptide coding sequence stalls in the presence of high tryptophan, preventing binding of Rho (purple hexagon) to the nascent RNA; this results in expression of the downstream genes. When tryptophan is low, the ribosome completes synthesis of the leader peptide (red line) and releases the RNA, which allows access of Rho and Rho-dependent termination. (c). Chloramphenicol-dependent translational control of the *cat* gene. In the absence of chloramphenicol, the leader peptide coding sequence is efficiently translated, and the SD sequence of the downstream *cat* gene is sequestered in an inhibitory structure. Low concentrations of chloramphenicol cause the ribosome translating the leader peptide to stall, which unfolds the mRNA structure and allows access of a second ribosome to the SD of the downstream gene.

whereas in the *E. coli trp* system, the translating ribosome senses charged tRNA^{Trp}; in both cases, the molecular sensors directly monitor their biochemical substrates while carrying out their normal biological reactions.

Regulation of RNA structure by RNA-binding proteins

A number of systems have been described in which binding of a regulatory protein to a leader RNA affects formation of an intrinsic terminator. These proteins can act negatively to stimulate termination (i.e., the terminator is inactive in the absence of the protein, binding of which promotes termination) or positively to increase readthrough of the attenuation site (i.e., the terminator is active in the absence of the protein, binding of which prevents termination and allows synthesis of the full-length transcript). The best characterized system in which a protein promotes terminator formation is the *Bacillus subtilis trp* operon, which

(a) Protein causes attenuation: *B. subtilis trp*



(b) Protein causes readthrough: *E. coli bgl*

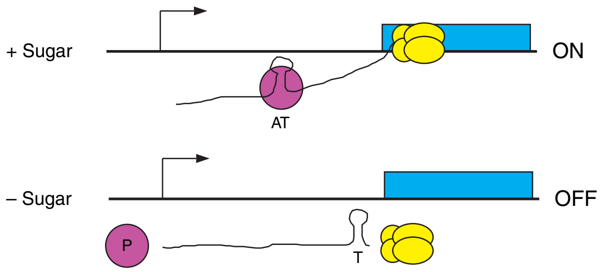


Figure 2 Regulation of transcription attenuation by RNA-binding proteins. (a) Protein-dependent attenuation in the *Bacillus subtilis trp* operon. When tryptophan levels are low, the *trp* operon leader RNA folds into an antiterminator element (AT) that allows RNAP (yellow ovals) to proceed past the attenuation site and transcribe the downstream gene (blue box). High tryptophan allows TRAP protein–tryptophan complex (pink circle) to bind to the leader RNA; TRAP sequesters sequences necessary for antiterminator formation, which allows formation of the terminator helix (T) and repression of downstream gene expression. (b) Protein-dependent antitermination in the *Escherichia coli bgl* operon. In the presence of the substrate sugar, the BglG RNA-binding protein (pink circle) binds to and stabilizes the antiterminator element (AT), which allows transcription to continue past the termination site. In the absence of the substrate sugar, BglG protein is phosphorylated (P) and unable to bind the RNA; the terminator helix (T) forms, and transcription of the downstream gene is repressed.

encodes gene products involved in tryptophan biosynthesis (**Figure 2**(**a**)). The *trp* leader RNA terminator helix is relatively unstable, and its formation is normally prevented by folding of the RNA into the more stable antiterminator structure, which includes residues necessary for terminator helix formation. Binding of the regulatory protein, designated TRAP, sequesters a region of the RNA that participates in formation of the antiterminator element and allows the less stable terminator helix to form, which causes termination. The RNA-binding activity of TRAP requires association with tryptophan, which serves as a corepressor. Termination therefore occurs when tryptophan is abundant, as described above for the *E. coli trp* operon, but in this case the molecular mechanism for sensing tryptophan availability involves binding of tryptophan to the regulatory protein rather than monitoring of charged tRNA$^{\mathrm{Trp}}$ during leader peptide translation. Charging of tRNA$^{\mathrm{Trp}}$ is sensed indirectly in *B. subtilis* through a second regulatory protein, designated anti-TRAP, which is expressed in response to a decrease in charged tRNA$^{\mathrm{Trp}}$ (via the T-box mechanism; see below) and antagonizes TRAP activity, allowing increased *trp* operon expression. This dual measurement of both free tryptophan and charged tRNA$^{\mathrm{Trp}}$ is similar to that observed in *E. coli*, although different molecular mechanisms for sensing the effector molecules are employed.

Just as the *E. coli trp* and *pyr* operons use leader peptide attenuation regulatory mechanisms, the *B. subtilis trp* and *pyr* operons both use an RNA-binding protein to control transcription termination. The *B. subtilis pyr* system is similar to the TRAP system, but has an added complexity in that the PyrR regulatory protein (in the presence of pyrimidine nucleotides as corepressor) binds to and stabilizes a leader RNA element that sequesters sequences necessary for formation of the antiterminator. Binding of PyrR prevents antiterminator formation and allows the less stable terminator helix to form. The PyrR-binding site therefore serves as an 'anti-antiterminator element', because it antagonizes the antiterminator element. This differs from the TRAP system, in that TRAP directly sequesters sequences involved in formation of the antiterminator without stabilizing an alternate RNA element. The reliance of both the *E. coli trp* and *pyrB* mechanisms on coupling of transcription and translation, while the analogous mechanisms in *B. subtilis* instead use an RNA-binding protein to modulate expression, may reflect differences in the efficiency of coordinating transcription and translation in these two organisms (and by extension, their close relatives).

RNA-binding proteins can also promote antitermination. Systems of this type utilize leader RNAs in which the terminator helix is more stable than the competing antiterminator, so that termination is the default state that occurs in the absence of protein binding. Binding of the protein to the RNA stabilizes the antiterminator element, which sequesters sequences that would otherwise participate in formation of the terminator helix. An example of this type of system is provided by the *E. coli bgl* operon, which is involved in utilization of β-glucoside sugars (**Figure 2**(**b**)). The BglG protein acts as the regulatory protein, and its RNA-binding activity is induced when the substrate sugar is present. The RNA target of the BglG protein includes residues necessary for formation of the terminator helix, so that BglG binding prevents termination and allows expression of downstream genes involved in sugar utilization. Unlike the TRAP or PyrR proteins, BglG does not directly bind the effector molecule but is instead dephoshorylated by BglF, the transporter for the sugar substrate, when the sugar is present. Dephosphorylation of BglG by BglF allows BglG to dimerize, which is required for RNA-binding activity. BglF therefore serves as the sensor of the effector molecule, and transmits information about effector availability to BglG via phosphorylation, in a manner analogous to that used by two-component regulatory systems in which phosphotransfer between a sensor kinase and a response regulator allows transmission of a regulatory signal from one protein to another. Several sugar utilization operons in *B. subtilis* are regulated by mechanisms similar to the *bgl* system.

Control of termination by RNA–RNA interactions

The ability of RNA molecules to base-pair with other RNA molecules provides another mechanism by which the structure of a leader transcript can be modulated, and RNA–RNA interactions have been exploited in a variety of regulatory systems. While most *trans*-acting regulatory RNAs in bacteria affect translation or stability of the target mRNA, binding of a *trans*-acting RNA to the nascent RNA can also affect the ability of the targeted region of the transcript to fold into terminator or antiterminator structures, thereby affecting attenuation or readthrough.

The best-characterized example of this type of mechanism is the T-box system, which is widely used in Gram-positive bacteria to control genes involved in amino acid metabolism, including aminoacyl-tRNA synthetase, amino acid biosynthesis, and transporter genes; as noted above, this mechanism also regulates synthesis of the anti-TRAP regulatory protein. Genes regulated by the T-box mechanism exhibit a complex set of conserved structural and primary sequence elements in their leader regions, which include a terminator and competing antiterminator. Embedded at a specific position within this array of conserved elements is a single codon that matches the anticodon of a tRNA that belongs to the amino acid class relevant to the gene product encoded in the coding sequence(s) located downstream of the terminator. For example, the *B. subtilis tyrS* gene, encoding tyrosyl-tRNA synthetase, contains a UAC tyrosine codon at the appropriate position. Binding of the matching uncharged tRNA (e.g., tRNA$^{\mathrm{Tyr}}$ for *tyrS*) to the leader RNA, which is determined by pairing of the leader region codon with the anticodon of the tRNA, is required to stabilize the antiterminator element by pairing of the four unpaired residues at the 3′ end of the tRNA with residues located in a bulge within the antiterminator (**Figure 3**(**a**)). The interaction of the appropriate uncharged tRNA with the nascent leader transcript therefore prevents formation of the terminator helix, and results in increased transcription of the downstream genes. The cognate charged tRNA (e.g., tRNA$^{\mathrm{Tyr}}$ aminoacylated with tyrosine) can also interact with the codon region, but is unable to stabilize the antiterminator because of the presence of the amino acid at the 3′ end of the tRNA. Codon–anticodon pairing is therefore responsible for selection of a particular tRNA species as the effector for a particular transcriptional unit, and pairing between the tRNA acceptor end and the antiterminator is responsible for discrimination between the uncharged and charged forms of that tRNA. The ability of the cognate charged tRNA to interact with the codon but not the antiterminator results in inhibition of binding of the effector uncharged tRNA, so that the true molecular signal is the relative amounts of uncharged and charged species of a specific tRNA. The cell therefore modulates expression of genes involved in converting a particular tRNA class from its uncharged to its charged state, in response to the substrate–product ratio.

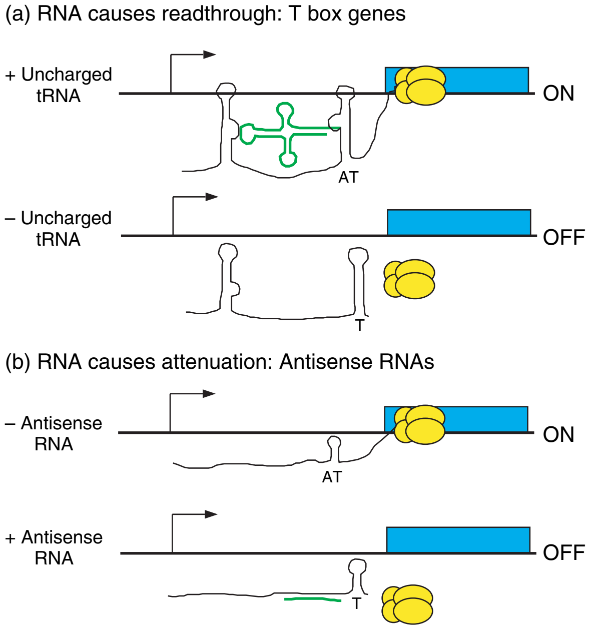

(a) RNA causes readthrough: T box genes
+ Uncharged tRNA
ON
AT
– Uncharged tRNA
OFF
T
(b) RNA causes attenuation: Antisense RNAs
– Antisense RNA
ON
AT
+ Antisense RNA
OFF
T

Figure 3 Regulation of transcription attenuation by *trans*-acting RNAs. (a) tRNA-dependent antitermination in the T-box system. When levels of a specific uncharged tRNA are high, the tRNA (green cloverleaf) binds to the nascent transcript of the gene that senses that tRNA; binding of the tRNA stabilizes an antiterminator element (AT), which allows RNAP (yellow ovals) to transcribe the downstream gene (blue box). When levels of the specific uncharged tRNA are low (i.e., the tRNA is predominantly in the charged state), the leader RNA folds into the terminator helix (T), and transcription of the downstream gene is repressed. (b) RNA-dependent attenuation by antisense RNAs. In the absence of the antisense RNA, an antiterminator element (AT) forms, and RNAP proceeds through the attenuator site and transcribes the downstream gene. Binding of the antisense RNA (green line) sequesters sequences necessary for antiterminator formation, and causes formation of the terminator helix (T) and repression of downstream gene expression.

The tRNA–leader RNA interaction and tRNA-directed antitermination have been demonstrated *in vitro* using purified components, which indicates that no factors other than the leader RNA itself are necessary for specific recognition of the cognate uncharged tRNA. The T-box mechanism, like systems that utilize leader peptide translation (e.g., *E. coli trp*), monitors the charging of a specific tRNA class to control leader RNA structure;

however, while leader peptide transcription attenuation systems monitor only availability of the appropriate charged tRNA, by using a translating ribosome, the T-box system monitors both charged and uncharged tRNA charging, by using direct binding of the tRNA to the nascent RNA transcript.

Several systems have been described in which binding of a *trans*-acting small RNA is proposed to modulate leader RNA structure to promote transcription termination. These systems, which have been analyzed primarily in plasmids, utilize *cis*-encoded antisense RNAs that bind to their target transcripts to, in some way, facilitate formation of the terminator helix, usually by destabilization of a competing antiterminator element (**Figure 3**(**b**)). Mechanisms of this type utilized more extensive base-pairing interactions between the regulatory RNA and its target. This role of small RNAs, while so far applicable only to *cis*-encoded RNAs, adds to the diversity of regulatory mechanisms that noncoding RNAs can affect in bacteria.

Metabolite-binding regulatory RNAs

A number of systems have recently been described in which binding of a small molecule to the nascent RNA transcript results in an RNA structural rearrangement that determines whether the transcript folds into an intrinsic terminator helix or a competing antiterminator structure. In most of these systems, the leader RNA includes a 5′ region that serves as an effector-binding 'aptamer' domain, which in isolation can specifically recognize the cognate molecule. Binding of the effector usually sequesters a segment of RNA that would otherwise participate in forming the antiterminator element (**Figure 4**(**a**)). The effector-binding domain therefore serves as an anti-antiterminator that prevents formation of the antiterminator, and therefore allows termination. This type of mechanism is generally used to regulate genes involved in uptake or biosynthesis of the effector molecule, and represents a type of feedback repression. A few systems show an opposite arrangement, whereby binding of the effector results in formation of the antiterminator, and promotes synthesis of the full-length transcript; in systems of this type, the effector is a substrate of the regulated pathway. Systems in which the nascent RNA directly senses the regulatory signal (a small molecule, a small RNA, or a change in physiological condition such as temperature) have been termed 'riboswitches'. The metabolite binding riboswitch RNAs exhibit highly specific recognition of their cognate effector molecules, which include vitamins, cofactors, nucleotides, amino acids, and metal ions, as well as an affinity for their target molecule appropriate to the physiological concentration of the effector. As in the T-box system, the effector-dependent RNA conformational change and transcription termination response can be reproduced *in vitro* in the absence of other cellular factors, indicating that the RNA transcript encodes all features necessary for the regulatory mechanism.

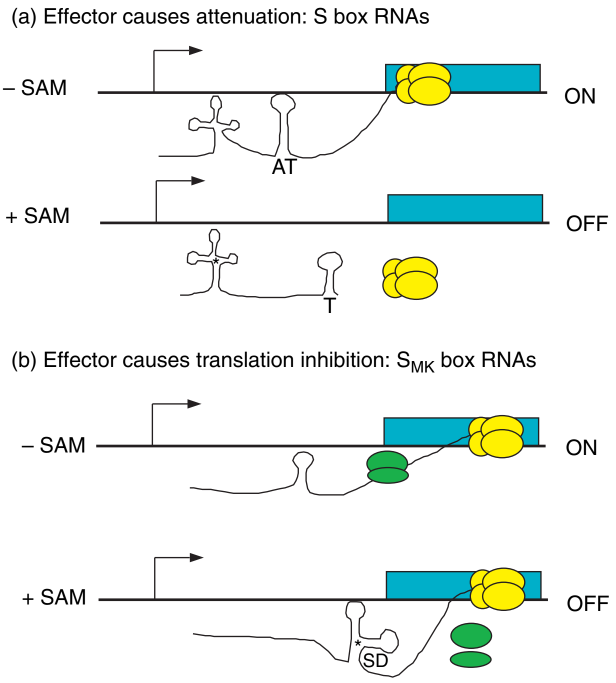

(a) Effector causes attenuation: S box RNAs
– SAM
ON
AT
+ SAM
OFF
T
(b) Effector causes translation inhibition: S$_{MK}$ box RNAs
– SAM
ON
+ SAM
OFF
*
SD

Figure 4 Regulation of transcription attenuation or translation initiation by riboswitch RNAs. (a) SAM-dependent transcription attenuation in the S-box RNAs. In the absence of SAM, the antiterminator element (AT) forms in the leader RNA, allowing RNAP (yellow ovals) to proceed through the termination site and transcribe the downstream gene (blue box). In the presence of SAM (∗), an RNA structural rearrangement causes formation of the terminator helix (T), resulting in attenuation of transcription. (b) SAM-dependent inhibition of translation initiation in the S$_{MK}$-box RNAs. In the absence of SAM, the SD region of the transcript is available for binding of the ribosome (green ovals), and translation of the downstream gene occurs. Binding of SAM (∗) to the leader RNA causes a structural rearrangements that results in sequestration of the SD region, preventing ribosome binding and downstream gene expression.

Reiterative transcription

As noted above in reference to the *E. coli pyrB1* operon, NTP abundance can affect the processivity of RNAP. The *B. subtilis pyrG* gene represents a system in which limitation for an NTP not only affects pausing but also results in nontemplated addition of residues to the transcript, in a process termed reiterative transcription. In this system, the transcript initiates with three G residues, followed by a C residue. When CTP is abundant, transcription proceeds through this region, and terminates at an intrinsic terminator prior to the start of the *pyrG* coding region. Limitation for CTP results in stalling of RNAP during synthesis of the 5′ region of the transcript, and RNAP stalling causes slippage relative to the template DNA and incorporation of a series of extra G residues into the transcript. RNAP eventually escapes from this site and continues transcription into the leader region. The resulting transcript now contains a longer run of G residues that are capable of pairing with C and U residues normally found on the 5′ side of the terminator helix. This pairing causes formation of an antiterminator element that is not directly encoded in the DNA template, and arises only from the reiterative transcription event. Reiterative addition of G residues, triggered by starvation of RNAP for CTP, therefore promotes synthesis of the full-length transcript, and *pyrG* expression, when cells are limited for pyrimidine nucleotides. This mechanism, like that of the *E. coli pyrB* operon, utilizes the transcription complex itself to monitor availability of its NTP substrates, but the mechanistic consequences of specific NTP limitation differ in the two systems.

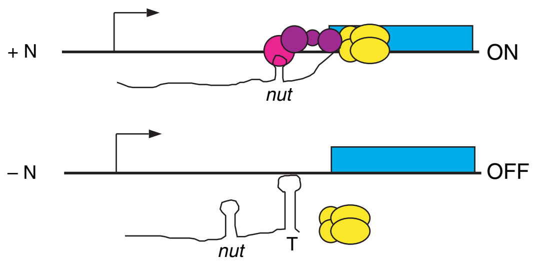
Regulation of Termination by Modulation of Transcription Complex Activity

All the systems described above involve changes in the structure of the nascent transcript, which arise as the result of interaction of other factors with the RNA, or through changes in the RNA sequence that occur during transcription. Another major class of transcription termination control systems does not utilize structural changes in the nascent RNA but instead involves changes in the behavior of the transcription machinery itself. These systems can modulate the processivity of RNAP or the ability of the termination machinery (i.e., Rho factor) to access the transcription elongation complex.

Protein-dependent changes in transcription complex processivity

The classic example of a system in which binding of a protein to a nascent transcript causes the transcription elongation complex to ignore downstream termination sites is provided by the bacteriophage lambda N-mediated antitermination mechanism. The phage-encoded N protein is responsible for the transition between the earliest stage of phage gene expression (during which only N and another regulatory protein, Cro, are synthesized) and the next stage of gene expression. N binds to specific sites (designated *nut* sites, for N utilization) on transcripts initiating at both the leftward (P_L) and rightward (P_R) major promoters. Once bound to these sites, N nucleates assembly of a complex of host-encoded proteins (the *nus* factors, for N usage substance) that interact with RNAP (**Figure 5(a)**). NusA protein binds first, and is sufficient for antitermination at sites close to the *nut* site; formation of a stable complex requires the addition of the remaining factors (NusB, NusG, and ribosomal protein S10). Assembly of the complete complex results in a more processive transcription elongation complex with reduced pausing and reduced termination activity at both Rho-dependent and intrinsic terminators, and this complex remains intact during transcription of long segments of DNA distal to the modification site. This effect has been termed processive antitermination because it allows readthrough of multiple termination sites that are encountered in succession as the transcription complex moves along the DNA.

(a) Protein-dependent changes in RNAP: lambda N



(b) RNA-dependent changes in RNAP: *put*

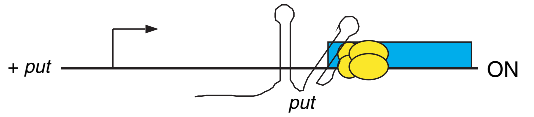


Figure 5 Changes in processivity of the transcription elongation complex. (a) In the bacteriophage lambda N system, N protein (pink circle) binds to the *nut* site on the nascent transcript and recruits additional factors (purple circles) that together modify RNAP (yellow ovals) to a complex that is resistant to transcription termination signals; this allows transcription of downstream genes (blue box). In the absence of N, the *nut* site is unoccupied and RNAP stops at termination signals that prevent downstream gene expression. (b) Bacteriophage HK022 uses the *cis*-acting *put* RNA element to modulate the processivity of RNAP, which results in readthrough of termination sites and expression of downstream genes. As the *put* site is always present in the RNA, its antitermination activity appears to be constitutive.

Bacteriophage lambda uses a second protein-dependent antitermination mechanism to promote the transition to late gene expression, mediated by the phage-encoded Q protein. In contrast to N, Q is a DNA-binding protein that interacts with the nontemplate strand of the DNA in a transcription complex paused just downstream from the late gene promoter. Q-directed antitermination also differs from the N system in that only a single host-encoded factor, the NusA protein, is required. Both the N and the Q systems are conserved in related lambdoid phage.

The lambda N antitermination system also serves as the target of a protein-directed termination system, in which a related phage, designated HK022, encodes a protein (Nun) that is related to N but interferes with N-mediated antitermination, causing failure of the N system and abrogation of the lambda lytic cycle. Nun binds to the *nut* sites on the nascent lambda transcripts and, in complex with the same Nus factors that are utilized by N to promote antitermination, instead promotes transcriptional arrest and termination. It is important to note that Nun does not inhibit N-mediated antitermination only by competing with N for binding to *nut* sites, but instead directly promotes termination at sites immediately downstream from the *nut* sites even in the absence of N. The biological role of the Nun system appears to be to promote HK022 propagation in cells coinfected with lambda, and HK022 utilizes a different class of antitermination mechanism to carry out its own life cycle (see below).

A different mode of protein-dependent modulation of the transcription elongation complex is represented by the RfaH system, which is found in a number of enteric bacteria. RfaH protein interacts with the nontemplate strand of the DNA on the surface of a transcription elongation complex paused at specific *cis*-acting sequences, designated *ops* sites. RfaH remains stably associated with the transcription elongation complex, and increases its processivity in a manner similar to the N and Q systems. This results in readthrough of potential termination sites in the downstream genes, and thereby stimulates synthesis of longer transcripts and expression of the downstream coding sequences. The RfaH system differs from the phage-encoded systems in that no cellular factors other than RfaH are required. RfaH-dependent antitermination is important for a number of processes involved in virulence in *E. coli* and its relatives.

RNA-dependent changes in transcription complex processivity

As noted above, phage HK022 utilizes the Nun protein not to mediate antitermination during expression of its own genome, but rather to prevent N-mediated antitermination by a coinfecting lambda phage. As for other lambdoid phage, antitermination is required for HK022 to advance into the lytic cycle, but in this case *cis*-encoded elements within the nascent transcript are responsible for readthrough of the early termination sites. Transcription originates from two divergent promoters (as described for lambda), and RNA elements (designated *put* sites) located downstream from the two promoters act in *cis* to promote readthrough of downstream termination sites and allow expression of genes located distal to the terminators (**Figure 5**(**b**)). The *put* sites are each comprised of two stem-loop elements, both of which are required for antitermination activity. These RNA elements are sufficient to promote antitermination in the absence of any HK022-encoded protein factors, and no host-encoded factors are required, in contrast to the lambda system. The *put*-encoded RNA elements interact directly with the β'-subunit of RNAP, and specific mutations in β' block *put*-mediated antitermination (and HK022 growth) without obvious effects on *E. coli* transcription or lambda growth. HK022 *put*-dependent antitermination appears to be constitutively active, as the *put* sites are always present in the nascent transcripts; this differs from the lambda N system, which allows regulation of downstream gene expression in response to N availability. Although the detailed mechanism of *put*-mediated antitermination remains to be elucidated, it appears that its function is similar to that of N protein in that it promotes both reduced pausing by the transcription elongation complex and processive antitermination.

Interference with Rho binding

Rho protein is the key factor in Rho-dependent transcription termination, and the requirement for interaction of Rho with the nascent transcript at *rut* sites provides a target for regulation. Binding of other factors to the transcript can affect the ability of Rho to access its binding sites on the RNA. The clearest example of a regulatory mechanism of this type is provided by the *E. coli tna* operon, encoding tryptophanase, which is involved in the utilization of tryptophan as a source of carbon and nitrogen. The tryptophanase coding region is preceded by a leader region that includes both a Rho-dependent termination site and a short peptide coding region (**Figure 1**(**b**)). When tryptophan levels, are low (conditions under which *tna* operon expression is repressed), the Rho-dependent termination site in the leader region is active, and transcription is attenuated. Under these conditions, translation of the leader peptide coding sequence within the leader RNA proceeds normally, and the ribosome and nascent peptide are released. When tryptophan levels are high, signaling a need for *tna* operon expression, a ribosome translating the *tnaC* region in the nascent transcript stalls and occludes the *rut* site within the leader region; Rho binding is prevented, and synthesis of the full-length transcript occurs, which allows production of tryptophanase. Tryptophan-dependent stalling of the ribosome during *tnaC* translation depends on specific features of the *tnaC*-encoded peptide, which in the presence of free tryptophan interacts in *cis* within the exit channel of the translating ribosome to inhibit peptidyltransferase activity, thereby preventing translocation. The requirement for free tryptophan to promote stalling of the ribosome–nascent peptide complex provides the sensitivity to tryptophan concentration necessary for an appropriate physiological response, since degradation of tryptophan is advantageous only when tryptophan is highly abundant.

Rho-dependent termination can also be regulated by the abundance of Rho protein itself. This sensitivity to Rho availability is utilized in autogenous regulation of *rho* gene expression. The *rho* gene contains several Rho-dependent termination sites, and synthesis of the full-length transcript is repressed when the cellular concentration of Rho protein is high. This represents a classical autorepression response that makes use of the biological function of the regulated protein to control its own synthesis.

Effects of Premature Termination on Upstream Gene Expression

While termination in a leader region is normally considered to control expression of coding sequences located downstream of the termination site, by determining whether the transcript includes those downstream regions, it is also possible for termination mechanisms to affect expression of genes located upstream of the termination site, in a process termed retroregulation. The classical example of this type of effect is observed in bacteriophage lambda, where N-mediated antitermination not only promotes transcription of genes downstream from the termination sites but also results in downregulation of the *int* gene, which is located upstream of one of the N-regulated termination sites. The *int* gene encodes the integrase necessary for integration of the lambda genome into the host chromosome, and its repression when N is active is a component of the switch between the lysogenic and lytic cycles. In the absence of N, the *int* transcript terminates at the intrinsic terminator site located at the end of the *int* coding sequence. N-mediated readthrough of this termination site results in synthesis of an extended transcript that now includes a new element (designated *sib*) that is sensitive to cleavage by the endoribonuclease RNase III. RNA cleavage at the *sib* site triggers the degradation of the mRNA region 5′ to the *sib* site by exonucleolytic degradation from the 3′ end released by RNase III, which results in a decrease in *int* mRNA levels. N-mediated antitermination therefore causes decreased stability of the *int* transcript, despite the fact that *int* is encoded upstream of the termination site targeted by N. It is likely that this type of regulatory effect occurs in other polycistronic transcripts in which the regulated terminator is located between coding sequences, especially since the presence of the helix of an intrinsic terminator often stabilizes transcripts by inhibiting access of the RNA degradation machinery (see below). Extension of the transcript by antitermination separates the 5′ coding region from the terminator element, which can provide additional targets for endoribonuclease cleavage and RNA destruction.

Regulation of mRNA Stability

The net amount of an mRNA transcript in a cell at any given time arises from the combined effects of transcript synthesis and transcript destruction. Changes in the stability of a particular RNA can have as great an effect on expression of the encoded gene product(s) as changes in the synthesis rate of that RNA. RNA degradation in *E. coli* occurs through the combined activity of a series of 3′–5′ exonucleases, and endonucleases that cleave within the RNA to provide new 3′ ends for attack by the exonucleases. Structure at the 3′ end of a transcript, as occurs when the 3′ end of the RNA is generated by an intrinsic terminator, inhibits access of the 3′–5′ exonucleases. Polyadenylation of the transcript by poly(A) polymerase (the product of the *pcnB* gene) provides a site for 3′–5′ exonuclease recruitment. While no 5′–3′ exonuclease activity has been found in *E. coli*, this activity is present in other bacteria, including *B. subtilis*. In either case, elements at the 5′ end of the transcript have been shown to affect mRNA stability, by affecting recruitment and activity of ribonucleases that attack there or at other sites on the RNA. Modulation of transcript stability can occur by binding of factors and/or structural rearrangements that affect accessibility of the RNA to ribonucleases. As noted above, premature termination of transcription can determine whether the transcript includes a binding site for a ribonuclease. Translation and mRNA degradation are also intimately intertwined, as binding and processivity of a ribosome influence the susceptibility of the mRNA to ribonucleases.

Mechanisms of mRNA Degradation

In *E. coli*, the endonuclease RNase E is the major initiator of degradation of full-length transcripts that contain intrinsic terminators at their 3′ ends. RNase E nucleates assembly of a complex, designated the RNA degradosome, that includes polynucleotide phosphorylase (PNPase, a 3′–5′ exoribonuclease), an ATP-dependent RNA helicase (RhlB), and enolase (a metabolic enzyme that may serve as a scaffold); poly(A) polymerase is also associated with the complex. RNase E binding requires several unpaired nucleotides at the 5′ end of the mRNA, and exhibits a preference for RNAs with a 5′ monophosphate (generated by other cleavage reactions or by removal of the 5′ pyrophosphate that is normally found at transcription initiation sites). Other cellular endonucleases can participate in further cleaving the RNA products, and polyadenylation, which is stimulated by endonuclease cleavage, plays a major role especially in degradation of RNAs with structural elements at the 3′ end. As noted above, *B. subtilis* contains an additional ribonuclease (RNase J1) with 5′–3′ exonucleolytic activity that may be especially important for degradation of RNAs that are protected from 3′ to 5′ exonucleases.

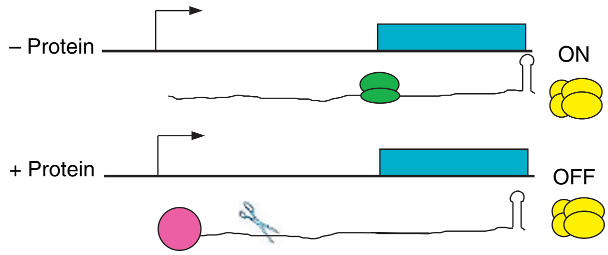
Regulation of Transcript Stability by Interactions with the mRNA Target

The crucial role of events at the 5′ end of the mRNA for initiation of the degradation process provides a target for regulation by modulation of 5′ end structure or accessibility. Sequences throughout the mRNA can also provide targets for endonucleases, and blocking the accessibility of these cleavage sites, through specific binding or by occupancy of the mRNA by translating ribosomes, can have major effects on the stability of the mRNA.

Regulation of mRNA degradation by RNA-binding proteins

A number of systems have been described in which binding of a protein to a transcript has a major effect on the lifetime of the transcript in the cell, and therefore on the expression of genes encoded within that transcript. This effect can be direct (by binding of the protein at a position that overlaps a site for recognition by a ribonuclease, which results in occlusion of the RNase binding site and stabilization of the transcript) or indirect (by interfering with translation initiation, thereby reducing protection of the mRNA by translating ribosomes; see below). The RNase E protein provides a clear example of a case where binding of a protein has been shown to directly affect mRNA stability (**Figure 6**(**a**)). RNase E represses its own synthesis by binding to a complex structural element in the 5′ UTR of its mRNA; this binding results in cleavage and subsequent destruction of the mRNA, and reduced RNase E synthesis. This autoregulatory mechanism takes advantage of the function of the gene product to maintain appropriate cellular levels.

(a) Protein causes mRNA degradation: *E. coli rne*



(b) sRNA causes mRNA degradation: *E. coli* RyhB

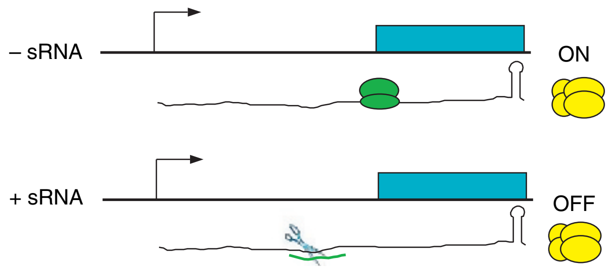


Figure 6 Regulation of mRNA degradation. (a) Protein-dependent RNA degradation in the *Escherichia coli rne* gene. When levels of the regulatory protein (RNase E, the product of the *rne* gene) are low, the RNA transcript is stable and efficiently translated by ribosomes (green ovals). High abundance of RNase E (pink circle) results in binding to the mRNA and increased degradation (scissors). (b) sRNA-directed mRNA degradation. In the absence of the sRNA, the target mRNA is stable and is efficiently translated. Binding of the sRNA to the mRNA by partial complementarity results in cleavage and rapid degradation of both the sRNA and its mRNA target.

RNA-binding proteins can also cause destabilization of a transcript by promoting binding of ribonucleases. An example of this type of mechanism is provided by the CsrA protein of *E. coli*, which controls a variety of carbon metabolism genes by affecting either mRNA degradation or translation initiation. CsrA binds to a helical element at the 5′ end of its target mRNAs, and CsrA activity is controlled by two regulatory RNAs (CsrB and CsrC) that contain an array of CsrA binding sites and therefore titrate CsrA away from its normal targets. A second regulatory protein, CsrD, controls the abundance of the CsrB and CsrC regulatory RNAs. Binding of CsrD to the CsrB and CsrC RNAs results in their rapid degradation, mediated in part by RNase E. CsrD therefore acts as a specificity factor that directs degradation only of its designated CsrB and CsrC RNA targets. This complex system allows rapid changes in CsrA activity, and therefore in target gene expression.

RNA-mediated changes in RNA stability

As noted above, the CsrB and CsrC regulatory RNAs indirectly affect mRNA stability by controlling the availability of the CsrA regulatory protein, which in turn affects mRNA stability or translation of other transcripts. A growing number of small regulatory RNAs (sRNAs) have recently been identified that affect the stability of other mRNAs, without their effect being directed by a regulatory protein (**Figure 6**(**b**)). These RNAs directly promote mRNA degradation (often with concomitant self-degradation) or interfere with translation initiation and therefore indirectly affect mRNA stability via the absence of translating ribosomes. Regulatory RNAs of this class (unlike CsrB and CsrC) interact with their targets by base-pairing, and may be encoded in *cis* (from the opposite strand of the same DNA region as the target) or in *trans* (from some other location in the chromosome). *Cis*-encoded regulatory RNAs are completely complementary to their targets (i.e., they are antisense RNAs), while *trans*-encoded regulatory RNAs are partially complementary, and often have multiple targets within the cell. In most cases, target RNA destabilization by *cis*-encoded sRNAs requires RNase III, which cleaves long double-stranded RNA regions, while RNA degradation by *trans*-encoded sRNAs has in several cases been shown to involve RNase E. For example, the RyhB sRNA, which is induced in response to limitation for iron, promotes degradation of a set of mRNAs encoding proteins that bind iron, allowing more efficient utilization of limiting supplies. The activity of *trans*-encoded sRNAs (like RyhB) is also often dependent on the RNA-binding protein Hfq, which acts as an RNA chaperone to enhance the RNA–RNA interaction, at least in part by modulating RNA folding to promote base-pairing between the two RNA partners. Both *cis*-encoded and *trans*-encoded sRNAs are usually degraded in concert with their target RNAs. Although destabilization of the target mRNA is the most common effect of sRNAs, the GadY sRNA in *E. coli*, which regulates genes involved in the response to low pH, stabilizes its mRNA targets and therefore increases expression.

Global Changes in the RNA Degradation Machinery

There is increasing evidence for modulation of degradosome composition and activity, especially in response to cellular stress. RNase E activity can be inhibited by two proteins, designated RraA and RraB, which cause alterations in the stability of many cellular mRNAs. Exposure to cold shock in *E. coli* also results in alteration of the degradosome. Starvation for amino acids activates several toxin–antitoxin systems that stimulate global degradation of ribosome-associated mRNAs, liberating resources for adaptation to the starvation state. Other general effects on translation affect overall mRNA stability, via effects on ribosomal occupancy. It seems likely that a large variety of regulatory events may target RNA degradation, given the complexity of the process and its importance to the cell.

Regulation of Translation

Translation of an mRNA provides another opportunity for regulation of gene expression. In bacteria, translation can begin as soon as the RBS has appeared within the 5′ region of the transcript emerging from the transcription elongation complex. Transcription and translation are usually temporally coupled, and the efficiency with which an mRNA is recognized by the translational machinery is variable and depends on primary sequence and the presence of structural elements. Translational elongation can also be modulated by elements within the RNA or *trans*-acting factors that influence ribosome processivity. Translational efficiency can in turn affect the stability of the mRNA by affecting accessibility to endoribonucleases, as noted above.

Regulation of Translation Initiation

In bacteria, translation initiation usually involves initial binding of the 30S ribosomal subunit, initiation factors, and initiator tRNA to the RBS, which is comprised of the Shine–Dalgarno (SD) sequence (a 5-nt sequence with the consensus GGAGG that is complementary to a region at the 3′ end of 16S rRNA within the 30S subunit) and the AUG initiation codon that specifies binding of the initiator tRNA; GUG and UUG codons are also recognized, albeit at lower efficiency. Translation initiation in bacteria differs from that in eukaryotic cells in that multiple translation initiation regions can be present on a single transcript, which allows the existence of polycistronic mRNAs from which multiple coding sequences can be independently translated. Binding of the translation initiation complex requires that the RBS be accessible, with no structure in the immediate region. Modulation of transcript structure in the RBS region therefore provides an opportunity for regulation that can be effective both while transcription is proceeding and after transcription has been completed.

Regulation of translation initiation by RNA-binding proteins

A number of systems have been described in which binding of a protein to the RBS region of an mRNA causes inhibition of translation initiation. As noted above, this inhibition may result not only in decreased translation of the mRNA but also in decreased mRNA stability, as a decrease in ribosome occupancy often causes degradation of the transcript. This type of mechanism is clearly illustrated in ribosomal protein genes in *E. coli*, where binding of one ribosomal protein encoded in a ribosomal protein gene operon results in sequestration of the RBS for the first gene in the operon, and decreased translation of the first coding sequence (**Figure 7**(**a**)). Expression of the

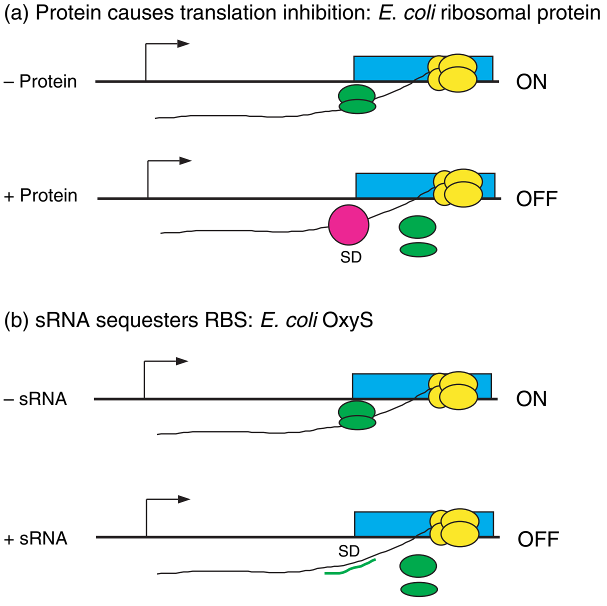

(a) Protein causes translation inhibition: *E. coli* ribosomal protein
– Protein
ON
+ Protein
OFF
SD
(b) sRNA sequesters RBS: *E. coli* OxyS
– sRNA
ON
+ sRNA
SD
OFF

Figure 7 Inhibition of translation initiation by RNA-binding proteins or sRNAs. (a) Sequestration of the SD region in *Escherichia coli* ribosomal protein genes. When the levels of the regulatory protein are low, the SD region of the target gene (blue box) is available for binding by the ribosome (green ovals), and translation occurs. When levels of the regulatory protein are high, the protein (pink circle) binds to an RNA element that overlaps the SD region, and ribosome binding is inhibited. (b) Inhibition of translation initiation by an sRNA. In the absence of the sRNA, the SD region of the target gene (blue box) is available, which allows binding of the ribosome and translation initiation. Binding of the sRNA (green line) sequesters the SD region and prevents ribosome binding.

downstream genes in that operon is also repressed, by a combination of translational and mRNA destabilization effects. Reduced translation of the downstream genes is likely to be due to translational coupling, where availability of the downstream RBS is dependent on unfolding of the mRNA by a ribosome translating the upstream coding sequence; interference with translation of the upstream region allows the transcript to fold into a structure in which the downstream RBS is sequestered. Inhibition of translation can also result in transcriptional polarity, where cryptic Rho-dependent transcription termination sites are activated by the absence of translating ribosomes. Together, these effects result in low expression of all downstream coding sequences, mediated by direct translational inhibition of only the first coding sequence.

Regulation of translation initiation by sRNAs

Accessibility of an RBS can readily be modulated by binding of a regulatory RNA, which can be encoded in *cis* (in which case the sRNA is perfectly complementary to its mRNA target) or in *trans* (in which case complementarity is usually imperfect, and Hfq is often used to enhance binding). Sequestration of the RBS region represents the simplest regulatory action of sRNAs, as binding of the sRNA to the RBS is sufficient to prevent ribosome binding, and recruitment of RNases by formation of a specific RNase binding site is unnecessary (although transcript destabilization is a likely indirect outcome of translational repression). There are many examples of sRNAs that repress translation, including a variety of plasmid replication control systems (which usually involve *cis*-encoded sRNAs), and the *trans*-encoded OxyS RNA, which is involved in the oxidative stress response in *E. coli* (**Figure 7**(**b**)). More rarely, translation initiation can be induced by an sRNA, as is the case for induction of expression of the *rpoS* gene, encoding the major stationary phase sigma factor in *E. coli*, by the DsrA sRNA. The *rpoS* mRNA contains a self-complementary region that encompasses the RBS, so that binding of the ribosome is blocked in the native transcript by an inhibitory helix. Binding of the DsrA sRNA to a region that includes the 5′ portion of this inhibitory helix results in unfolding of the helix, and liberation of the RBS region so that translation initiation can occur. As noted above, effects on translation initiation also result in changes in mRNA stability, amplifying the translational effect.

Regulation of translation initiation by RNA structural rearrangements

Most of the classes of regulatory events in which RNA structural rearrangements are used to control gene expression at the level of premature termination of transcription (discussed above) can also be used to regulate at the level of translation initiation. The major difference between transcription attenuation systems and translation initiation systems is that in the latter systems the crucial regulatory helix acts to sequester the RBS of the regulated gene, rather than serving as the helix of an intrinsic terminator. For example, the processivity of the ribosome during leader peptide translation in systems like the *E. coli trp* operon determines whether a terminator or competing antiterminator helix forms in the nascent transcript. In an analogous translational control system, like that of the Gram-positive *erm* and *cat* genes (which encode resistance to erythromycin and chloramphenicol, respectively), leader peptide translation modulates whether the RBS of the downstream coding sequence is sequestered in a helix that prevents translation initiation (**Figure 1**(**c**)). Expression of these genes is induced by exposure to subinhibitory concentrations of the antibiotic, which promotes stalling of the ribosome during leader peptide translation. The stalled ribosome sequesters a region of the transcript that would otherwise form an inhibitory helix that occludes the RBS of the downstream resistance gene, allowing translation of the downstream gene to commence. The stalled ribosome also stabilizes the downstream region of the mRNA, by a mechanism that may include blocking the activity of the 5′–3′ exonuclease found in this group of organisms. In both the antibiotic resistance genes and systems like the *E. coli trp* operon, the translating ribosome serves as the sensor for the regulatory signal (the antibiotic or charged $\mathrm{tRNA}^{\mathrm{Trp}}$); a major difference is that termination control systems like the *E. coli trp* operon require precise coupling between transcription and translation, so that the transcription elongation complex can respond to the translational signal as it reaches the termination site, while translational control systems like *erm* and *cat* can exert their effect either while transcription is taking place or after transcript synthesis is complete.

Riboswitch systems can also operate at the levels of premature transcription termination or translation initiation, and in several systems the same effector binding domain is used for both regulatory mechanisms. The major difference between termination and translational riboswitch systems is the position of the final inhibitory helix, the formation of which is determined by effector binding. In transcription attenuation systems, this helix is usually located at least 30 nt upstream of the start of the regulated coding sequence, and includes the run of U residues in the transcript that together with the helix serve as the intrinsic termination signal. In contrast, the inhibitory helix in translational control systems (which lacks the U residues) is positioned to include the RBS (or at least the SD sequence) in the 3′ side of the helix, so that helix formation occludes the RBS and prevents translation initiation. There are also examples where binding of the effector (e.g., the uncharged tRNA in T-box genes) stabilizes a helix that includes sequences that would otherwise base-pair with the SD (i.e., the ‘anti-SD’ sequence), so that effector binding results in increased accessibility of the RBS and increased translation. In most riboswitch systems, the effector binding domain is separate from the regulatory domain, which allows an easy transition between transcription termination control and translational control. An exception to this is the *S*-adenosylmethionine (SAM)-responsive S_{MK} box, which regulates SAM synthetase genes in lactic acid bacteria; in this case, the SD–ASD pairing region is an intrinsic component of the SAM-binding element (**Figure 4**(**b**)).

RNA thermosensors represent the simplest class of systems for regulation of translation initiation. In these RNAs, the RBS is sequestered into a helical structure that is stable under normal growth temperatures. An increase in temperature results in unfolding of the helical structure, and accessibility of the RBS to binding of the 30S ribosomal subunit. Regulatory elements of this type are obviously well suited to genes that are involved in cellular responses to increased temperature, and are found in certain heat shock genes including the *E. coli rpoH* gene, which encodes the heat shock sigma factor. The *rpoH* mRNA is present in the cell during normal growth conditions, but translation is inhibited by the thermosensor element. A sudden temperature shift melts the inhibitory helix. This results in a rapid increase in sigma protein levels, which triggers the general heat shock response (including increased transcription of *rpoH* itself, which is preceded by a promoter recognized by RNAP containing the heat shock sigma). Restoration of the normal growth temperature inactivates the *rpoH* transcript which refolds into the inactive helical structure, blocking further synthesis of the heat shock sigma factor and facilitating a return to steady-state conditions. Similar RNA thermosensors have been identified in heat shock genes of other bacteria, and have also been shown to regulate the *Listeria prfA* gene, which encodes a key regulator of virulence gene expression. In this case, the temperature change is used by the organism to sense movement into the host, which signals a requirement for induction of the virulence response.

Repression of translation initiation by alterations in the translational machinery

The *E. coli infC* gene, encoding translation initiation factor IF3, provides an interesting example of using the function of a protein in an autogenous regulatory mechanism. IF3 plays a role in discrimination of authentic translational start codons. The initiation codon for infC itself is a highly unusual AUU codon, which is poorly recognized by initiation complexes containing IF3. Therefore, if IF3 is present in saturating amounts, translation of the *infC* gene is inhibited, and no additional IF3 is made. A reduction in IF3 abundance is signaled by the accumulation of initiation complexes lacking IF3. These defective complexes fail to discriminate against the *infC* mRNA, which results in increased IF3 synthesis until the complexes are again saturated. This regulatory mechanism is absolutely dependent on the presence of the AUU codon, which is found in *infC* genes of many bacteria, suggesting that the regulatory mechanism is conserved.

Regulation of Translation Elongation or Termination

The shift from translation initiation to elongation requires addition of the 50S ribosomal subunit, and loss of initiation factors. Once this step has occurred, the translating ribosome becomes highly processive, and is generally effective at displacing RNA structural elements or bound proteins. As noted above, translating ribosomes can affect the stability of the transcript, by preventing access of ribonucleases, or the structure of the mRNA, which can affect transcription termination or translation initiation for downstream coding sequences.

Codon bias effects

Processivity of the ribosome during translation is dependent on the supply of charged tRNA entering the A site of the elongation complex. The presence of a codon for which the corresponding charged tRNA is at low abundance can cause a transient pause in translation; this is the basis for leader peptide transcription attenuation systems, as described above. Not surprisingly, codons for which the corresponding tRNAs are not abundant in a given organism are not heavily used in that organism. These rare codons can generally reduce translational efficiency, and bias against rare codons has been noted in highly expressed genes, such as those encoding ribosomal proteins. The presence of rare codons can also be used in regulation of gene expression. The best-characterized example of this is provided by the *bldA* gene in *Streptomyces coelicolor*, which encodes a tRNA specific for the UUA leucine codon. This codon is rarely used in the *Streptomyces* genome, with a strong bias for genes involved in developmental processes. The *bldA*-encoded tRNA is dispensable for normal growth, but a *bldA* mutant is defective in secondary metabolism and sporulation because of the presence of the corresponding UUA codons in these genes. Regulation of *bldA* expression can therefore affect a variety of genes containing UUA codons, resulting in a shift in the gene expression pattern; this effect can be further amplified if *bldA*-regulated genes include regulators that affect other groups of genes.

Programmed frameshifting

While translating ribosomes are usually resistant to structural features in the mRNA, certain RNA structural elements can promote ribosome pausing; elements of this type are important for alterations in normal coding, such as programmed frameshifting and insertion of unusual amino acids like selenocysteine. Frameshifting occurs when a ribosome shifts from its standard triplet decoding of an mRNA to a movement by 1 nt either forward (+1 frameshift) or backward (−1 frameshift). The ribosome then resumes translation, and decodes a different set of triplets on the same mRNA. Programmed frameshifting is a genetically encoded phenomenon that directs ribosomal frameshifting at a specific site, and in a specific direction. It can promote synthesis of multiple products from the same mRNA, as in the *E. coli dnaX* gene, which encodes two subunits of DNA polymerase, one of which is derived from a shift in reading frame that is dependent on both a structural element and a primary sequence element that promotes pausing of the elongating ribosome. A frameshift event is also used in autoregulation of the *E. coli prfB* gene, which encodes a translation termination factor, ribosome release factor 2 (RF2). The *prfB* gene is highly unusual in that it contains a UGA nonsense codon early in the coding sequence. Translation termination at UGA codons employs RF2, and efficient termination at this site (and failure to synthesize additional RF2) occurs when cellular levels of RF2 are high. A decrease in RF2 abundance results in ribosome stalling at the UGA codon; this stall increases the probability of a shift of the ribosome into an alternate reading frame, which allows synthesis of the full-length RF2 protein. Like a variety of other autogenous control systems, *prfB* autoregulation makes direct use of the function of the RF2 protein in translation termination to modulate expression of its gene.

Conclusion

The variety of posttranscription initiation gene regulation events that occur in bacterial systems demonstrates the versatility of the gene expression machinery. Each step in gene expression represents a potential target for regulation, and the investigation of multiple gene systems in a broad range of experimental organisms broadens our appreciation of what remains to be discovered.

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Prions

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Glossary

abnormal PrP Prion protein (PrP) molecules that are associated with pathology. A final terminology is pending and PrPsc, PrPres, or PrPd is used. Abnormal PrP may possess templating properties necessary to nucleate toxic or infectious proteinaceous structures.
amyloid A deposit of aggregates of PrP molecules resistant to proteolytic degradation and enriched in neuronal tissue.
BSE Bovine spongiform encephalopathy.
CJD The abbreviation for Creutzfeldt–Jakob disease of humans. It is the most common prion disease in humans; it occurs sporadically or can be inherited as in fatal familial insomnia (FFI) or Gerstmann–Sträußler–Scheinker (GSS) syndrome.
prion Name given to certain unconventional pathogens, suggesting their apparently proteinaceous and infectious nature.
PrP The abbreviation for the mammalian prion protein encoded by the PRNP gene on human chromosome 20. PrP-encoding genes are found in many mammalian species.
PrPc The abbreviation for the cellular PrP; its function is not yet unambiguously defined.
PrPd Abbreviation referring to disease-associated PrP. This nomenclature has recently been introduced to include all PrP molecules observed when prion disease is diagnosed with respect to PrP.
PrPres Abbreviation indicating PrP molecules that are resistant to experimental degradation with proteinase K (PK) or cellular metabolic proteolysis. Besides PK-resistant PrP molecules, PK-sensitive PrP molecules have also been associated with infectious prions.
PrPsc Abbreviation for 'prion protein\–scrapie,' consisting of PrPc molecules with an infectious capacity. PrPsc may contain PrP molecules resistant or accessible to experimental or naturally occurring proteolyitc degradation.
TSE Abbreviation for transmissible spongiform encephalopthies (TSE), a synonym for prion disease. Examples are scrapie of sheep, kuru and CJD in humans, BSE of cattle, which is etiologically related to variant CJD (vCJD) in humans and chronic wasting disease (CWD) of cervids (deer and elk).
vCJD Abbreviation for variant CJD, etiologically linked to infectious prions from BSE and may represent a zoonosis. Intra- and interspecies transmission of CJD is feasible with a few exceptions.

Abbreviations

AHSP	alpha hemoglobin-stabilizing protein
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt–Jakob disease
CWD	chromic wasting disease
EEG	electroencephalogram
ELISA	enzyme-linked immunoabsorbent assay
FDC	Folicular dendritic cells
FFI	fatal familial insomnia
GSS	Gerstmann–Sträußler–Scheinker
IHC	immunohistochemistry
LRS	lymphoreticular system
ORF	open reading frame
PCR	polymerase chain reaction
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PrP	prion protein
PrPc	cellular PrP
PrPd	disease-associated PrP
PrPsc	prion protein\–scrapie
2-DE	two-dimensional gel electrophoresis
WB	western blot

Defining Statement

Prion diseases or transmissible spongiform encephalopathies are infectious neurodegenerative disorders. Infectivity is associated with prions, proteins associated not only with infectious diseases but also with the inherited and sporadic disorders. The true nature of prions is unknown. However, molecular variants of the host cell-encoded prion protein can not be excluded as constituents of a prion. The protein-only hypothesis is consistent with current understanding of mammalian prions. Reliable evidence for an inheritable nucleic acid in prions so far remained elusive.

Prion Diseases

Disease

Prions have been widely accepted as the infectious etiological agent of widespread prion diseases characterized by brain damage with a spongy appearance. Hence, they are called transmissible spongiform encephalopathies (TSE). The best known of these is scrapie in sheep, which was first described in 1759. Intensive research on TSE began in the 1950s when Gajdusek discovered that human kuru was related to cannibalism and to the ritual use of diseased brain, and thus the suspected kuru to be transmissible.

In the early stages of TSE research, a conventional transmissible agent was suspected to be involved and therefore the disease was classified as a lentiviral ('slow virus') infection. Even in the 1920s, when Creutzfeldt and Jakob made their first pathological observations, there was a rumor that attempts were made to transmit the disease to animals, probably the rabbit.

However, there are no records of transmission studies until the successful transfer of kuru from humans to chimpanzees was reported by Gajdusek in the 1960s. After this, researchers in the United States and Europe set out to define the infectious agent. The search for prions in decisive experiments with ultracentrifugation and nucleic acid techniques began in the mid-1970s, coinciding with the introduction of hepatitis B virus research, which searched for particles in blood and led to the eventual detection of DNA and the infectious 42 nm hepatitis B virus Dane particle.

Despite numerous experimental efforts, no convincing virus-like agent or any kind of conventional pathogen with nucleic acid as the genetic material had been isolated, although highly sophisticated methods have been applied. Even with latest techniques in molecular biology at hand, the predicted and long-sought virus could not be identified. Therefore, the protein-only hypothesis should not be abandoned until the true nature of prions is known. Absence of evidence for a viral etiology is, strictly speaking, not evidence for the absence of a hidden viral component. Owing to the enigmatic nature of prions, Koch's postulates have been used to question prions as the postulates are not fulfilled. However, the biological characteristics of some viruses also do not meet these postulates.

The most important prion diseases are sporadic Creutzfeldt–Jakob disease (CJD) (sCJD) and variant CJD (vCJD), whereas genetic CJD, fatal familial insomnia (FFI), and Gerstmann–Sträussler–Scheinker disease (GSS) represent only 10–15% of cases.

Typically, sCJD affects patients in their 60s. Cases have a median disease duration of about 6 months. In contrast, vCJD cases are in their 30s or even younger and the duration of disease is prolonged up to 15 months. At disease onset, vCJD is characterized by psychiatric symptoms; later, vCJD cases develop dementia, ataxia, and myoclonus like sCJD cases. Magnetic resonance imaging (MRI) revealed differentiable brain images between sCJD and vCJD cases. Genetic cases of sCJD, like those associated with the E200K (glutamic acid replaced by lysine) PrP mutation, cannot be clinically differentiated from sCJD unless family history and genetics are established. FFI and GSS have atypical clinical symptoms, often go undetected, and require family history and genetics for positive diagnosis. FFI and GSS appear at younger ages, and the progression from first clinical symptoms to death may be delayed for years. Finally, iatrogenic CJD resulting from neurosurgery, corneal grafting, or dura mater implants are clinically indistinguishable from sporadic CJD.

Jakob and Creutzfeldt initially defined characteristics of the disease denoted as CJD. Human kuru, more so than scrapie, became the most striking disease to be linked to prions. Kuru victims were identified until 2000 because of a latent and persisting prion infection. Therefore, a long period of subclinical persistence of prions before the onset of disease existed. The mechanisms controlling pathogenicity are not completely known, and genetic variation associated with the host-encoded prion protein gene seems to act as a modifier of infection and disease. BSE of cattle emerged and its transmission into the human population was of great concern. The persistence of prions and prion strains is of concern in the case of BSE-human variant CJD (vCJD) transmission, since there is some worry that unique BSE strains might induce not only vCJD but also induce sCJD. On the other hand, even in geographical areas where scrapie is endemic, no increase in the incidence of human prion disease has been found. Notably, prions of scrapie differ biologically from prions of BSE.

Neurodegeneration and loss of neuronal cells are hallmarks and cause disease. Hypotheses to explain the neurodegeneration include the following: (1) prions may act alone as causative agents to deteriorate cells, (2) loss of the cellular PrP might be responsible, and (3) perhaps abnormal PrP or its deposits are cytotoxic. Most diagnostic procedures, especially postmortem immunohistochemistry (IHC), detect only late stages of prion infection and disease. Within the group of human sporadic CJD, clinical symptoms vary and can be correlated with prion protein glycotyping in Western blots (WBs) and DNA polymorphisms of the host's gene encoding the prion protein. Noninvasive MRI and electroencephalogram (EEG) became promising tools for the detection and classification of disease. To understand disease progression, behavioral studies assessing alterations of the circadian rhythm and sleep–wake cycles in human prion diseases are rare but can be performed to a certain degree in animal models. Only FFI could be assessed with respect to these parameters. Most important for humans, the classical CJD clinically differs from vCJD, a fact that lends initial support to the hypothesis that vCJD was a transmission of BSE into humans in the mid-1990s and therefore represents a zoonosis. As of October 2007, 201 vCJD cases have been reported worldwide. Clinically, vCJD is distinguishable from classical forms of sporadic CJD, genetic prion diseases such as FFI and GSS, or CJD associated with octarepeat and point mutations. Other clinical symptoms are sometimes shared by prion disease. For example, elk suffering from chronic wasting disease (CWD) experience cachexia (wasting) and salivate extensively in a way comparable with the distinct clinical human MV2 CJD or vCJD phenotype.

Neurology and neuropathology clearly identify not only classical prion diseases but also newly emerging ones such as variant CJD in humans or CWD of elk. Clinical symptoms are reliable diagnostic markers and the experienced clinician is able to distinguish on the basis of the disease phenotype which human prion disease is being examined. With similar diagnostic certainty prion disease of sheep, cow, elk and other animals can be identified by the experienced veterinarian. From the different diseases and their heterogeneous clinical and molecular characteristics, prion strains have been postulated and were eventually defined.

Epidemiology

TSEs are widespread in the animal kingdom. Scrapie is almost ubiquitously found, whereas BSE had a limited global spread, mostly in Europe. Prion disease of deer and elk is growing in North America and Canada, and scrapie persists in indigenous areas.

Human kuru occurred in the past and has almost been eradicated, with the last cases reported in 2000. These slow progressors were old and their resistance or reduced susceptibility has been genetically correlated to the type of their prion protein gene.

As to sporadic CJD, the inherited FFI, and GSS, no significant increase of the incidences has been observed worldwide; about 1.4–1.6 CJD cases/1 000 000 were observed in 2005.

After BSE was introduced into food chains and no transmission barrier prevented BSE prions from infecting humans, a major problem emerged. How efficient the infection in humans might have been and what will happen if prions persist over a long period of time and subclinically in individual persons are still unanswered questions. Predictive epidemiological models began with little data on a disease with long incubation times. Therefore, early predictions had a large variance. With the ongoing epidemic for vCJD, the reduction in incidence and increase in the number of data points have improved the predictive ability of subsequent mathematical models. Even sophisticated statistical models could not compensate for the lack of reliable data on persistence and activation mechanisms acting in prion infection. Relating BSE cases to human vCJD is hampered by the incorrect numbers of BSE cases reported and the low numbers of cases of vCJD; both make statistical analyses difficult. Therefore, examination of the the coincidence of endemic areas for BSE and vCJD favors BSE as the etiological agent of vCJD until we have solid evidence for any other cause. Incidence also discriminates sCJD from inherited CJD and vCJD, because epidemiology could correlate vCJD cases to BSE cases. When CJD incidences increase, one might suspect either new prions in disguise or improved surveillance. Although only few BSE cases were reported outside of Europe, they should not be neglected. Export of contaminated beef from Europe to Asia has been supposed to be a risk factor with an unforeseeable outcome. Fortunately, sufficient exposure to prions, called infection pressure, was reduced as risk-associated animal material was removed and should not enter food chains. There is no doubt that epidemiology depends on both the nature of prions and their biological behavior. Distinctive prion strains, persistence, and in apparent infection may veil authentic cases. To understand the mechanisms as to how prions are hidden or disguise themselves is a major goal in current prion research.

Pathogenesis

The pathology of a prion infection with progression to disease is attributed to pathological or abnormal PrP molecules. Deposition of PrP isoforms such as amyloids is a striking pathological trait of the disease and thus relevant.

Before the pathological deposits can be detected, morphological changes like clustered cavities described as vacuolization of the tissue are only seen in prion disease. Vacuolization and appearance of pathological PrP are distinct events that occur at different times after infection. Yet, the loss of biologically active PrPc might also contribute to the damage of neuronal cells. Whether or how they are linked on a molecular basis has not yet been worked out conclusively.

Clearance of abnormal PrP may be brought about by proteolytic enzymes but clearcut experimental data on such processes are not available. Because PrP molecules may be involved in signal cascades controlling cellular metabolism, morphological alterations might be the first sign of damage by prions.

Longitudinal studies in animal models or in infected cells are experimental approaches to define individual steps of prion disease, which is a difficult task because prions possess strain characteristics as mentioned in the section titled 'Disease'. Strains were defined on tropism, the pattern of local lesions, and their pathological phenotype can be dissociated from their molecular phenotype. Different strains may lead to different phenotypes, for example, disease and unawareness of the presence of different strains in a natural setting will lead to difficulty in interpreting results.

To further our knowledge on death and loss of neuronal tissue, basic research may yield further clues. Sometimes higher levels of abnormal PrP in brain can be found in experimental models like transgenic animals. Yet, these animals behave normally, do not present with neurological deficits, and seem only to be attacked by prions. Hence, it is difficult to initiate therapeutic measures as there is no early sign of disease. Disappointingly, therapeutic measures with candidate compounds like quinacrine have been initiated in humans but eventually failed. Deposition of prion protein in amyloidal structures may be responsible for toxic effects, rendering cells susceptible to cell death mechanisms. Certain PrP molecules may become neurotoxic by adopting distinct structures similar to PrP of octarepeat mutant prion disease, or recombinant genetically altered PrP molecules are toxic in permanently growing N2a neuroblastoma cells. Concomitantly, there may be a loss of physiological PrP, which would in turn also contribute to disease. In contrast to these results were surprising reports suggesting that PrP may confer neuroprotection. If PrP is convincingly identified as a positive functional survival factor in cells, then some current paradigms must be changed.

Data on a positive role of PrP in cellular physiology are not yet satisfactory, but transcriptomics and proteomics are promising tools to decipher cellular pathways in prion infection and subsequent deleterious processes. Experiments with PrP-deficient cell lines are most promising. They can be transfected with plasmids introducing PrPc, and changes brought about by normal PrP can be monitored. Alterations in the transcriptome and the proteome may provide insight as to how cells respond to this newly introduced PrP.

Experiments have produced evidence that PrP and its conformational transition are key to the pathogenesis of prion diseases. Several PrP intermediates have been identified but the exact conformational transition of a normal PrPc into malignant forms is not known. Presumably, mechanisms are involved that are based on molecular interactions with ligands and the thermodynamic stability of disease-causing alternate PrP conformations. A highlight in prion research is the emerging evidence that brain tissues from healthy individuals, human or animal, harbor PrP molecules prone to become abnormal, which thus explains sporadic prion disease as a stochastic process predicted from enzyme reaction kinetics. In line with these findings is the observation that mice genetically manipulated not to express the prion protein gene never showed signs of disease even if inoculated with prions. Suspected cofactors in mammalian cells or tissue are expected. Candidate molecules may belong to chaperones such as heat-shock protein 104, a protein required for conversion of the yeast prion-like protein Sup35, which is described in 'Structural components of prions' in more detail. How mammalian PrP isoforms in the cell act as positive or negative factors in the cellular physiology is still not clear, for example, whether they are involved in interactions with growth-regulating factors bcl-2 or whether they are part of signal cascades.

Prevention and Therapy

Conceiving prevention measures and interventions is the subject of intense clinical research.

Protective measures such as postexposure prophylaxis after iatrogenic transmission by meat consumption or eradication of horizontal spread, especially of CWD of elk, have to be developed. Excluding especially neuronal tissue as a source of infectivity was successful in reducing the risk of transmitting BSE. Use of recombinant growth hormone instead of hormone purified from cadavers was very successful and iatrogenic prion infections almost disappeared. This appears to be a situation reminiscent of acquired immunodeficiency syndrome (AIDS). Preventive measures were especially taken in neurosurgery where disposable instruments are used; concomitantly, efficient decontamination protocols have to be developed because prion infectivity tightly sticks to steel. Keeping livestock free of prions is hard to achieve as infectivity, transmissibility, and persistence of prions are still insufficiently understood.

Breeding and selection of sheep homozygous for a unique prion protein genotype (PRNP for humans and prnp for mice) encoding arginine and arginine at codons 134, 156, and 171 were expected to resist prion infection. However, persistent and subclinical infections with classical scrapie seem to prevail in these breeding stocks. As described in 'Strains', prions might occur in a mixture or 'swarm', assuming a variable prion population with one strain possessing higher replicative capacity than the others. There is no immediate answer as to how prions and transmission can be extinguished. The state of being prion- or BSE-free may be an impossibility. Sporadic CJD in humans, possibly also as scrapie in sheep and also BSE in cattle, exists and might emerge stochastically. To solve the problem in livestock, genetically manipulated PrP-deficient cows have been discussed to prevent sporadic prion disease.

Therapeutic measures in human prion disease have been conducted in a few sCJD cases with unsatisfying results, that is, at best a slight delay of death. The patients were terminally ill and the results of these studies are disputable because some compounds used are toxic. Quinacrine was hoped to be beneficial but this too eventually failed. In the hamster model, tetracycline derivatives delayed the onset of disease but there was no cure.

Reversible immunomodulation has been considered but may not be applicable due to an autoimmune response against the PrP that is not foreign to the host. Transgenic knockout mice with defects in complement genes showed a delay of prion disease. These experiments will not lead to a method of treatment but they were scientifically helpful since the role of the immune system and prions could be assessed. The most intriguing results were obtained with antibodies that recognized the cellular prion protein and decelerated prion disease. The monoclonal antibodies may not have 'neutralized' the prion itself as in a passive immunization against a virus. Instead, the antibodies may bind to the PrPc, thus, either interrupting precursor-like complexes of newly made abnormal PrP or preventing the deadly connection between PrPc and prions recruiting PrPc for toxic or infectious PrP aggregates. Thus, gene transfer of genetically engineered anti-PrP antibody has been suggested. Alternatively, genetically engineered PrP to titrate out intracellular, emerging abnormal PrP or exogenous prions presents an interesting possibility but experiments have thus far met only limited success. Gene therapy to keep prion infection and disease under control is theoretically feasible, but it is questionable as to whether it can ever be achieved. New concepts are well recognized, but gene transfer with viral vectors and local synthesis of PrP may be inefficient. It still must be clarified as to which vector is appropriate, for example, integration into the host genome with the accompanying disadvantages, or only extrachromosomal with transient expression.

The Agent

Nature and Origin

Prions are unique in infection biology. Their true nature is not known although numerous experimental efforts have been undertaken to unravel the structural composition of an infectious prion, its shape as a pathogen, and where its infectivity resides. Most experimental data are in favor of the protein-only hypothesis, postulating protein structure(s) as the heritable non-Mendelian component instead of a virus with coding nucleic acid.

However, evidence for a viral origin of prions was initially derived from the detection of nucleic acid in prion preparations. RNA from intracisternal A particles became a candidate but no sequences were reported whose origin could be linked to a virus genome. Infection experiments with CJD prions pointed to interference, a phenomenon known from retroviral infection. Interference occurs if one retrovirus inhibits or at least limits infection with a second retrovirus, for example, by blocking cellular virus receptors. This finding was indicative that prions with different infectious capacity exist and strains were postulated analogous to, for example, attenuated virus strains. Electron microscopy revealed in tissue and cells small 25 nm particles that were suspected to be a small prion virus with the size of parvoviruses or circoviruses. However, the proponents of a viral etiology have not yet produced any nucleic acid that can be unambiguously confirmed as the prion-specific nucleic acid. In contrast, nucleic acid molecules, such as noncoding polyanionic ribonucleic acid (consisting of poly A), as well as lipids, did promote to the formation of abnormal PrP in the protein misfolding cyclic amplification (PMCA) assay. Previous and recent ultraviolet inactivation experiments repeatedly excluded nucleic acid molecules larger than 200 nucleotides, leaving a possibility for a prion-associated small noncoding RNA like micro-RNA with a size of only 30–50 nucleotides. Experimental data obtained over years in transmission studies in transgenic mice and in PrP-folding assays with recombinant PrP or PrPc preparations still favor the protein-only hypothesis, that is, prion transmission via protein structures. In parallel to *in vitro* PrP conversion assays, newly developed cell culture assays might help to further define critical components for prion infection, prion formation, and prion transmission under defined experimental conditions. This approach will probably be successful at objectively and efficiently testing natural prion isolates or experimentally made prions.

Immune responses have very often helped to identify the nature and origin of pathogens, but it is not so in prion research because PrP is encoded by the host and is not foreign. Prion research began with a major surprise, confirming the PrP detected in infectious brain homogenates to be encoded in a cellular gene; it was denominated PRNP in humans and other species. The PRNP was identified and sequenced in many species, including those that are susceptible to prion disease. There is one open reading frame (ORF) coding for the PrP with a molecular size of approximately 30–35 kDa. Previously, other genes such as SINC for scrapie incubation period in mice had been described but they were ultimately shown to be identical with the PRNP.

To unveil function(s) of PrP, knockout mice were generated that have no functional PrP, and thus were called PrP 0/0. Most of these PrP-deficient mice strains did not suffer from an obvious physical or neurological defect so that no functional role of PrP could be deduced from them. Loss of PrP function, gain of PrP function, or toxicity by certain PrP molecules, each in its specific experimental environment, might have been compensated for in these animals. Most remarkably, in these animals, no progression to disease after infection with prions was observed. This finding paved the way to considering PrPc as a prerequisite for prion disease and propagation of prions.

A scheme of the ORF coding for the PrP as well as some biochemical and structural characteristics are depicted in **Figure 1**.

The polymorphism at codon 129 leads either to methionine or valine in the mature PrP. Point mutations are mostly missense mutations; nonsense mutations leading to shortened PrP as well as octarepeat insertion mutants are rare. All mutations are important when PrP structure and folding mechanisms are to be connected or when susceptibility and transmission of prion disease are investigated.

The primary amino acid sequence of PrP molecules renders them more or less prone to conformational transition. Any structural shift in one and the same PrP molecule might become relevant when it begins to turn into abnormal forms, which are eventually linked to neurodegeneration and synthesis of infectious transmissible prions. The propensity to adopt a certain molecular structure makes them targets for folding, unfolding, or refolding mechanisms that depend not only on thermodynamic stability but also on auxiliary or accessory cellular components. Comparable with the human PrP, a similar genetic heterogeneity is found in sheep and, to a lesser degree, in other species.

Certain topological traits will be discussed as the PrP as an endogenous cellular protein may represent one – perhaps the most important – component of a prion. PrP is posttranslationally modified by removal of the 5′- terminal

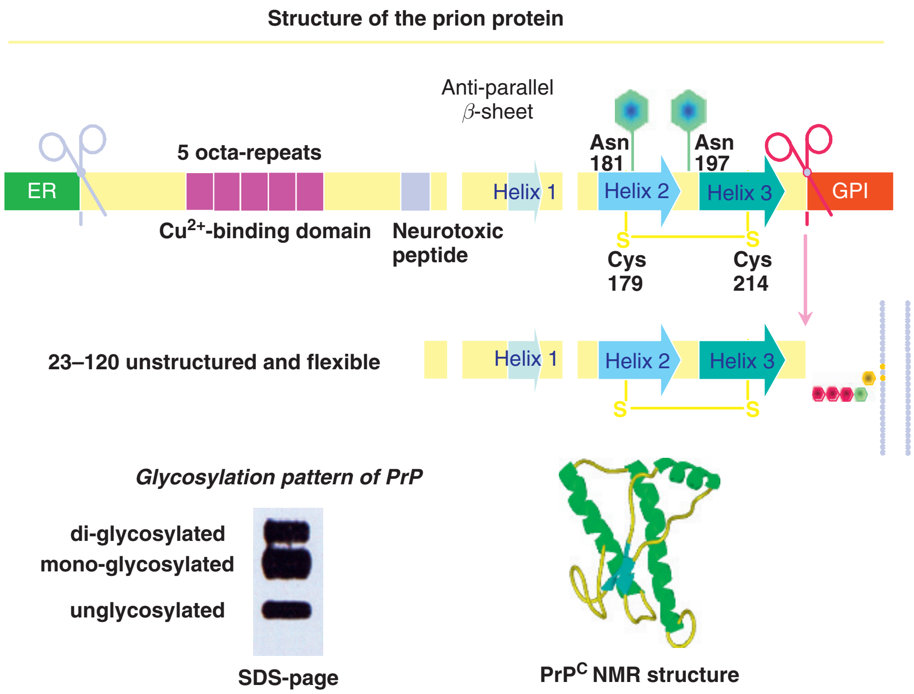

Figure 1 Schematic representation of the topography of cellular PrP. The signal sequence (ER) and the GPI anchor are proteolytically removed, and the proteinase K accessible sites are shown at amino acid 85–95. The octapeptide repeat, the neurotoxic region, and the regions consisting of helical and β-sheet structure, the Cys–Cys link and the N-glycosylation sites are also indicated. The 23–120 region is removed when cellular PrP is converted into the PK-resistant PrP often designated PrPres. The tripartite banding pattern of PrP seen in Western blots allows strain typing. NMR structure provides a three-dimensional image of a cellular PrP molecule.

leader sequence, by N-glycolsylation, and by fusion of a GPI anchor to become membrane-bound as shown in **Figure 1**. Crystallization experiments suggested a homodimeric PrP structure, but no data are available as to whether this PrP dimer exists in a living cell.

Despite extensive experimental efforts to define a physiological function for PrP, only copper binding brought about by the octarepeat motifs in the PrP is currently regarded as a possible function. Detrimental PrP molecules affecting intracellular trafficking can be produced in cells and, when incorrectly incorporated into cellular membranes, may damage cells. Topography of membrane-embedded PrP also has been discussed as a determinant for cytotoxicity or prion formation. How PrPc becomes resistant to intracellular proteolytic degradation, to gain a propensity to acquire a preponderance of β-sheet structures, and thereby becoming a precursor for abnormal PrP, is unknown but essential to understand the pathomechanisms of neurotoxicity, disease, and formation of prions. Unexpectedly, protecting functions, such as prevention of apoptosis, supporting growth, or participation in cellular signal cascades like the Fyn pathway, have been ascribed to PrP. Further investigations are to be expected and cell lines from PrP-deficient mice seem to be suitable tools to uncover new physiological tasks for PrP.

Altogether, these experimental findings on PrP are essential not only for the protein-only hypothesis but also for the loco-lesional (site where lesions appear) toxicity in prion disease.

Structural Components of Prions

Originally, brain specimens with suspected prions were analyzed and abnormal PrP could be detected. Converting the protein sequence into a nucleic acid sequence was the pioneering experiment in the identification of PrP as a cellular gene and not as a nonhost, pathogen-related protein.

Currently, hypotheses involving folding mechanisms have been suggested to explain the conversion of PrPc into a growing group of rogue, abnormal PrP species suspected to be associated with infection and disease. They are now designated as PrPd in order to include all novel abnormal PrP molecules that are present in diseased tissue and cells. Most abnormal PrP can be distinguished from the PrPc by treatment with proteinase K (PK). PK degrades PrPc, whereas abnormal PrP conformers resist this experimental condition. Examples of WB strain typing is given for hamster scrapie 263 K, human sCJD, and BSE from cattle in **Figure 2**(**a**) and a schematic representation of abnormal PrP is shown in **Figure 2**(**b**).

The three PrP bands represent the typical diglycosylated, monoglycosylated, and nonglycosylated PrP isoforms from brain of prion-diseased humans and animals. Normal isoforms exist in the brain and can become PK-resistant in the diseased brain as misfolding protects the PK-resistant PrP isoforms. Without PK treatment, PrPc appears as a smear because of the glycocylation.

After experimental digest with PK, which is called limited digestion, normal PrP completely disappears. If typical abnormal PrP is present, then three PrP isoforms form a prion-type-specific banding pattern with distinct molecular sizes, and variable quantities of each band can be recognized.

The often used nomenclature PrPres resulted from the experimental approach describing PrP resistant to PK. In contrast, PrPsens means PrP sensitive to proteolytic degradation. Definitions became more complex when variable accessibility of PrP molecules to proteolytic degradation was shown to exist.

Additional abnormal PrPres molecules have been detected even in the brain of non-CJD cases. These aberrant PrPres molecules could represent precursor type PrP convertible into those abnormal PrP molecules, eventually becoming part of prions.

Even more disturbing were recent findings suggesting that PK-sensitive PrP molecules were components of infectious prions. Sometimes PrPsc, originally derived from scrapie, is used as a synonym for infectivity. To sort out these variant PrP molecules, new technology like nanotechnology is required to further analyze single molecules.

As described above, prions seem to consist of abnormal PrP. However, detection of prion-related banding patterns is sometimes not possible and, unfortunately, the presence of abnormal PrP does not consistently imply that infectivity is definitely present or absent.

The contribution of a highly ordered structure of PrP in aggregates to infectivity might be compared with yeast prion-like protein Sup35 aggregates. Distinct aggregates consisting only of Sup35 proteins can propagate certain phenotypic traits by a non-Mendelian inheritance. This finding emphasizes that the protein-only hypothesis for mammalian prions should not be excluded because similar, even if not identical, epigenetic mechanisms in yeast exist. Because prion-like proteins in yeast have led to improved experimental approaches for mammalian prion proteins, the basics behind yeast prion-like proteins is briefly summarized. In baker's yeast, *Saccharomyces cerevisiae*, the translation termination factor Sup35 can exist in an aggregated state, resulting in read through of a nonsense UAA codon. This state is defined as PSI+ and is inherited in a non-Mendelian manner. Aggregated Sup35 protein is able to recruit newly formed nonaggregated Sup35 protein into an insoluble form. In contrast, soluble Sup35 protein terminates translation at stop codons like UAA and confers the PSI− (or wild type) state. This molecular behavior resembles that of the

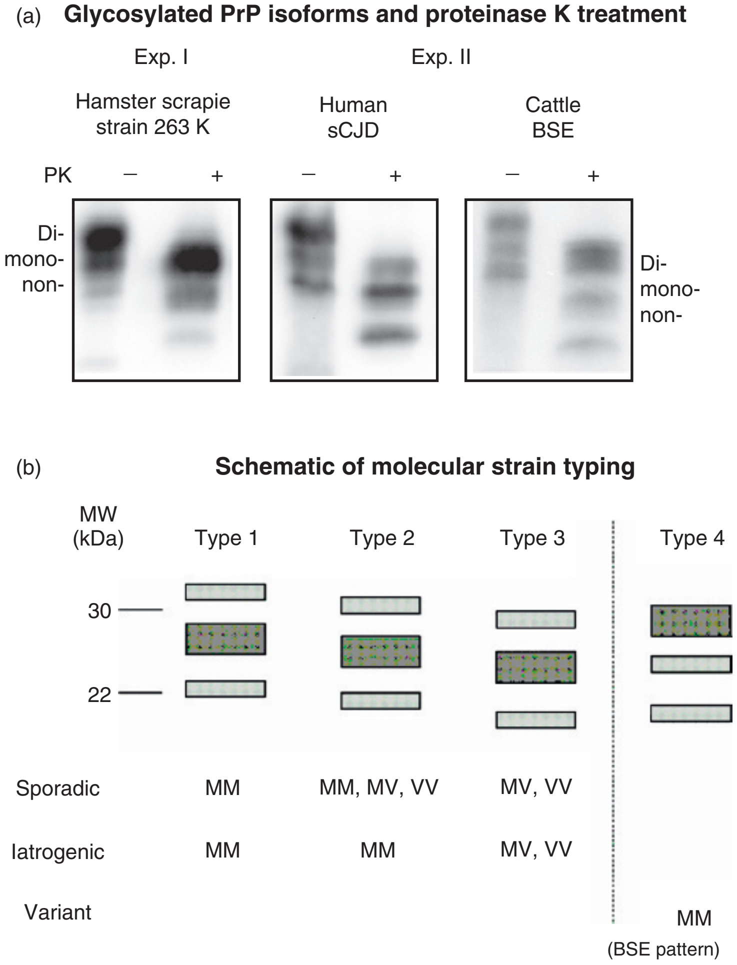

Figure 2 (a) Western blot of PrP from hamster scrapie 263 K, human sCJD, and BSE from cattle before and after experimental treatment with proteinase K (PK). Untreated PrP appears as a smear because of the carbohydrate residues affecting the migration behavior of PrP in denaturing gel electrophoresis. After PK treatment, the tripartite PrP banding patterns emerge and allow biochemical strain typing. (b) Strain typing by PK treatment and resolution of PrP into typical tripartite banding patterns. The type 1–3 banding patterns are found in individuals with either polymormphisms: methionine/methionine, methionine/valine, or valine/valine at codon 129. All vCJD cases have type 4 and are homozygous for methionine/methionine (MM and not MV or VV). Sporadic and the acquired iatrogenic prion disease forms are of types 1–3 and share genetic susceptibility associated with codon 129. The PrP type 4 banding pattern was observed only in human vCJD cases and in BSE indicative for BSE transmitted to humans. At present, the type 4 vCJD is only found in cases with MM homozygosity thought to confer higher susceptibility for prion infection and disease.

mammalian PrP where insoluble PrP aggregates can be formed, may become toxic, or form part of prions. In contrast, non-aggregated PrPc, whatever function it may have, exists inside and on the surface of cells. Experimentally, the PSI+ state and PSI− state can be differentiated easily in yeast cell culture. The correct translation termination, that is, the PSI− state leads to a red pigment in yeast strains containing a UAA nonsense mutation in *ADE2* because the mutated ade2-1 gene is terminated at the UAA mutation. In the PSI+ state, however, these mutants are characterized by white yeast cells. Instead of the termination of translation at the UAA, aggregates of the Sup35 protein are nonfunctional, do not recognize UAA, and thus suppress the nonsense UAA, leading to a read through of the ade2-1 mutation and yeast cells remain white. Aggregated Sup35 protein can be generated with recombinant Sup35 protein. The recombinant Sup35 protein was folded and aggregated in the test tube and these experimentally formed yeast prions could be ‘transmitted’ into yeast cells, changing the PSI- state of the recipient yeast cells into PSI+. These experiments showed unambiguously that the experimental transmission of the PSI+ state is independent of any nucleic acid as the information molecule and the results lend essential support to the protein-only hypothesis for mammalian prions.

PrP oligomerization can be forced under experimental conditions and unique PrP oligomers seem to be essential in propagating infectivity as shown in cosedimentation experiments seeking associations between protein structure and prion infectivity. Similar experiments might eventually explain why octarepeat mutant PrP is toxic but the suspected prions behind toxicity are difficult to transmit; this is remarkable in prion disease. Perhaps, specific misfolding is involved in infectious activity and the octarepeat mutant PrP is not able to adopt such a structure. Other oligomeric PrP structures may inhibit infection, and could suggest why Alzheimer's disease is a nontransmissible neurodegenerative disease, although its pathogenesis also involves atypical aggregation of cellular protein or processed parts thereof.

The spectrum of anticipated PrP with or without measurable infectivity again corresponds to prion-like Sup35 proteins in yeast, where incorrect or incomplete aggregates are crucial for transmitting the Sup35 phenotype PSI+ or PSI−. Together with mammalian strain determination, the yeast prion-like proteins provided a general model for strains, their internal structure depends on the respective proteins, and confer heredity in the absence of nucleic acid.

The role of auxiliary components such as carbohydrates, either covalently bound or covering prions together with adherent lipids, remains unclear. Carbohydrates on prions could mimic viral glycoproteins and might be indispensable for secretion, transport, or migration along neuronal and nonneuronal routes. Glycan residues may act as ligand(s) for cellular prion receptors and thus be a codeterminant for the uptake of prions into cells.

Despite extensive experimental efforts, no convincing prion-specific nucleic acid was found. However, small noncoding RNA species, such as small interfering- or micro-RNA or cellular RNA as binder to prions, cannot be excluded.

Infectious prions were inefficiently synthesized in first-generation conversion assays and in cell lines but never from recombinant PrP. This changed since autocatalytic PMCA became available. With this technique, prions can be replicated *in vitro* and the propagation of infectious prions was confirmed in transmission experiments. Highly purified cellular hamster PrP could sporadically convert into infectious prions in a PMCA assay, reminiscent of the acquisition of sporadic CJD *in vivo*. Recombinant PrP species are yet to be successfully converted *in vitro*, and whether they induce prion disease in transgenic mice is a matter of debate. Fine tuning of conversion assays and newly developed cell culture systems appear to be promising tools for solving such questions.

Only rough estimates regarding the infectivity related to abnormal PrP exist. Approximately 10^6 pathological, especially PK-resistant PrP isoforms were correlated to one infectious unit. This calculation may not be valid because PrP molecules associated with disease may even be PK sensitive and infectivity is still preserved. Determination of infectivity also depends on the site of prion inoculation. Among intracerebral, intraperitoneal, or oral modes, differences of 5 orders of magnitude exist. This tremendous difference illustrates that host factors besides PrP are involved, as will be described in the section titled 'Contribution of the host'. Dilution of prion inocula and titration in susceptible cell lines in a virus-like plaque assay may become feasible.

Besides bioassays, biochemical and physical techniques might resolve PrP structures in prions. X-ray diffraction analysis of crystallized PrPc extended nuclear magnetic resonance analyses, and suggested that PrP exists as a homodimer. Circular dichroism analyses uncovered dynamic structural changes of PrP molecules. The molecular shift from α-helical to β-sheet structures and specific domains such as peptide loops have been defined. Fluorescence-based techniques have been successfully applied to study the aggregation of PrP molecules or with ligands like monoclonal antibodies. Atomic force microscopy was instrumental in the description of amyloidal structures, and studies in molecular dynamics will complete the structural studies of PrP.

Prions are highly resistant to heat and disinfectants. Methods of disinfection that eliminate viruses do not efficiently eradicate prions. Strong acids or high concentrations of sodium hydroxide are required. Prion infectivity also resists formaldehyde and ethanol. To completely abolish infectivity, high temperatures, that is, 134–136°C for 20–60° min are needed.

Treatment with 98% formic acid reduces infectivity but improves IHC to detect pathological PrPres, which withstands experimental digestion with PK at concentrations from 10–100 μg/ml for 30–60 min. Most alarming was the transmission of prions via steel wires and surgical instruments. These facts explain why prions survive also in organs and result in human vCJD after meat consumption from cattle with BSE. Recipients of blood transfusions developed prion disease. These unsettling observations turned researchers' attention to body fluids such as saliva, feces, and urine in cervid prion disease. Latest results with urine in the hamster prion model have clearly shown that these body fluids and excrements contain infectious prions and are of concern because of horizontal transmission. PrP has also been found in soil, clay, or loam. Results on infectivity are now available and point to a sustained contagion in the environment.

Replication

The replication mechanism of mammalian prions has not been established, although replication of prion-like proteins in yeast represents a solid model. To generate PrP aggregates, molecular mechanisms are postulated to connect pathogenic PrP isoforms with the wild-type ones in models where seed PrP molecules are present and nucleation as well as folding and aggregation takes place. Toxicity and acquiring infectivity then follow. Reaction kinetics support such a scenario. Efforts to synthesize mammalian prions with the PMCA have been successful. Now, any factors rendering pathological PrP into prions in cells and tissue can be tested in the tightly controlled PMCA. In parallel, differential gene expression may help identify additional replication factors such as for conformational transition. Tracking low levels of infectivity, for example, from saliva, should be possible and help to assess this route of transmission. Furthermore, PMCA offers experimental approaches to study potential DNA or RNA sequences as binders to PrP. Similarly, aptamers of different biochemical origins might be used to define their role in prion replication. As a spinoff, aptamers might be found that distinguish PrP isoforms in diagnostic assays or eventually become candidate molecules for therapy. For instance, an aptamer could contribute to melting PrP aggregates during replication and render them susceptible to intracellular proteolysis, which would make such an aptamer suitable for therapy.

The PMCA resembles protocols established for yeast Sup35 *in vitro* conversion. The Sup35 approach considered a cycling process as crucial for aggregate formation and for propagation of phenotypic traits. If incorrectly aggregated, yeast prions may become nontransmissible agents, which might also explain why prions from certain prion diseases like GSS are difficult to transmit in animal models. GSS prions may not consist of distinct PrP aggregates, which because of how they aggregated are not infectious.

Strains

A few introductory comments are necessary to understand strains in prion research. The existence of prion strains has been deduced from differences among phenotypes, such as disease, lesion profile, biochemical typing, intra- as well as interspecies transmission, and host range phenomena. These attributes make prions virus-like, although a viral etiology is not proven. It is known that PrP is encoded in the prion protein gene of the host where the prion originates. This is a unique phenomenon in infection biology, in that a host cell-encoded gene product becomes part of a transmissible, infectious activity. Depending on the primary amino acid sequence, distinct PrP molecules are synthesized with individual characteristics affecting conformational transition and proteolytic degradation. Some pathogenic mutations and polymorphisms are presented in **Figure 3**.

If PrPc turns into abnormal PrP, it may adopt certain structure(s), and if it gains infectivity, it can be propagated as a prion strain. Strain characteristics seem to be inherited via the protein structure alone and there is no conclusive evidence for nucleic acid as an essential component for the propagation of a prion strain. After a prion is established as a strain, it seems that no nucleic acid-dependent process occurs either during transmission or later in the recipient, which is reminiscent of the propagation of strain characteristics by non-Mendelian inheritance in yeast. A protein-only mechanism that imprints strain specificity onto the cellular counterpart is confirmed for the Sup35 protein of yeast and cannot be excluded for PrP in mammalian cells.

Transmission of mink prion isolates into hamster was one of the essential experiments for defining the prion strains Drowsy and Hyper, the names of which refer to the clinical symptoms they induce in the recipient Syrian hamster.

Besides clinical symptoms for distinguishing strains, the PrP banding patterns became a reliable method for molecularly describing strains. The three protein bands represent one smaller PrP molecule without carbohydrate side chains, an intermediate one with one glycosylation

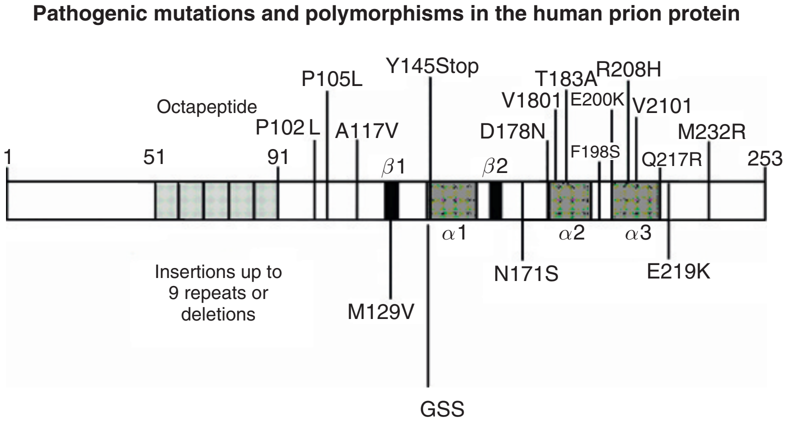

Figure 3 Mutations in the human PrP sequence. Amino acids are numbered 1–253. Numbers refer to point mutations, for example, the substitution of methionine by valine at codon 129. A stop codon is found at codon in GSS. Within the octapeptide regions, single repeats are either inserted or in rare cases deleted.

side chain, and a large molecule with two N-bonded glycosyl chains, with apparent molecular sizes in sodium dodecyl sulfate (SDS) polyacrylamide gels between 30 and 35 kDa and shift to 20–27 kDa after PK treatment since approximately 100 amino acids from about 253 are removed (**Figures 2**(**a**) and **2**(**b**)).

Therefore, prion typing based on abnormal PrP is very instructive with respect to strains for tracing them in tissue and cells and for determining what happens to a strain after transmission into a second host.

In fact, such experiments clearly demonstrated that BSE prions were transmitted from cattle to humans with vCJD both possessing the same prion strain of type 4 or type 2b according to two current classification protocols.

In experiments to explain how prions are transmitted between species, transgenic mouse lines were produced. In numerous experiments, it was shown that the PrP encoded in the recipient transgenic mouse controlled the outcome of transmission and preserved the inoculated strain. Some results were as expected, that is, strain characteristics were propagated as in the yeast model. However, other results were alarming because strain switch or strain splitting, indicative of the presence of more than one strain in one inoculum, was experimentally confirmed for BSE and vCJD prion transmission in human PrP producing mice. Prion strains preexist or may evolve even in short-term transmission experiments. Perhaps, a prion quasi-species exists and replication-prone or fit strains emerge faster and propagate more efficiently, similar to what is known for viruses. Adaptation, such as reduction of incubation time of strains, was detected when one prion strain like CWD was repeatedly passed in mice. This phenomenon is well known in virology where such selective adaptation mechanisms can lead to virus strains with a different host cell tropism or a modified virulence.

Strain typing was improved by PrP analyses in high-resolution two-dimensional gel electrophoresis (2-DE) and confirmed that strain-specific banding patterns were not lost during passage. Whether or not a strain switch can be identified or correlated to a 2-DE protein pattern has not yet been reported.

Using transgenic mice became a method of choice and humanized, bovinized, and ovinized transgenic animals have been made available. Lately, unrecognized BSE strains, such as bovine amyloidotic spongiform encephalopathy or atypical scrapie, have been identified by PrP typing and confirmed in transmission experiments.

The transmission of prions and the respective PrP pattern in tissue implied in most experimental settings the presence of prions comparable with most naturally occurring BSE cases. It can be noted that lack of detectable abnormal PrP concomitant with prion infectivity may be indicative of a strain persisting in a subclinical or a silent carrier stage.

New strains comparable with the sheep NOR 98 are most likely to emerge as current methods and assays for detecting disease-associated PrP or prions are superior to those applied in the past.

Strain typing defined by tropism is also one possibility for describing strains. Strains appear to disseminate inside a host on different routes. In contrast to the neurotropic murine ME 7 strain, strain RML prefers nonneuronal tissue in the periphery. Lymphotropism has been observed for vCJD, whereas BSE seems to travel on neuronal routes. Although speculative, tropism of prion strains might result in persistence or latency in a host.

Strain characteristics may also depend on where prions are produced and how they are posttranslationally modified. Glycosylation might determine the route where prions migrate in an infected host. Very often glycosylation supports the secretability and solubility of proteins in aqueous body fluids like immunoglobulins in serum.

MRI as a noninvasive method proved valuable for indirect typing of prion strains. The prion-induced lesion pattern in the brain of sCJD cases is distinguishable from vCJD and adds to the list of clinical phenotypes typical for sCJD versus vCJD.

Infectivity and Transmission

Entry

To gain entry into a host, tissue and cells should possess receptors for prions. The best studied receptor is the laminin receptor precursor molecule. It binds PrP and it is supposed that infectious prions cannot be internalized if this receptor is lacking. Other receptors such as cadherin or N-cam were discussed but additional data need to be compiled. Whether intracellular ligands, such as regulatory molecules, play a role after the uptake of prions is not known.

How precisely receptor molecules like carbohydrate receptors or lipids might mediate entry into tissue and cells by fusion processes is still not understood. Likewise, initial events in mucosal tissue or neuronal tissue after exposure to prions have not been sufficiently investigated.

Enteral invasion is far less efficient than the experimentally preferred intracerebral inoculation. Proteolytic degradation in the gastrointestinal tract is presumed to reduce prion infectivity. The gastrointestinal system, the lymphoreticular system, components of the immune system, and various tissues and cell types are candidate sites for the uptake and migration of prions from non-neuronal peripheral tissues into the peripheral and central nervous system. To study this, hamsters were orally inoculated and prions monitored by determining abnormal PrP along the route from intestinal tissue into the brain.

In sheep, elegant experiments were performed and showed the migration of prions, that is, abnormal PrP through the enteric nervous system to lymph node tissue. In human vCJD, prions are also suspected to enter via epithelial and immune-competent cells in the tonsils.

Kuru was caused by parenteral inoculation when prion-contaminated human brain was allocated to small lesions in the face or into eyes and nose for ritual reasons. Tonsils and tongue are proximal to neuronal tissue and made immediate infection possible. Intracerebral inoculation became the routine technique in experimental TSE to track down and investigate infectivity in animal models such as hamster, mice, and transgenic mice. Innovative cell culture systems are now becoming available together with easy tests for infectious prions from biological source material. If suitable cell lines for prions are available, then the search for prion receptor molecules will advance. Genetically altered cell lines rendered permissive or refractory to prions will considerably expand the fragmentary knowledge on prion receptors and ligands.

Persistence and Latency

Unfortunately, the initial steps in prion infection and the early stages of disease remain a mystery. Over a long time span, no physical or clinical evidence for prions is recognizable. It is conceivable that low levels of abnormal PrP molecules or abnormal PrP with a propensity for adopting pathogenically relevant intermediate structures are limited in the cell due to proteolytic clearing. Intriguingly, prions behave like persistent viruses, remaining latent long before (re)-activation by a trigger.

Infection of inbred mice strains with hamster or BSE prions was obscured as no prion disease was apparent and abnormal PrP was undetectable. However, transmission experiments unambiguously proved that infectivity was conserved in persistent prions. This is of major concern because silent prion carriers may be hidden in populations and may go undetected even with sensitive detection methods for abnormal PrP.

The long incubation period, on the average 5 years for BSE and up to 40 years in kuru, is characteristic of the unusual nature of prions and its distinctive inapparent stage between infection and terminal disease. The recent detection of unpredicted abnormal PrP molecules in healthy, non-CJD individuals is taken as evidence for a natural prevalence of certain prion-prone, abnormal PrP molecules. Such precursor PrP molecules were originally postulated from reaction kinetics and provided insight into the conformational transition of normal PrPc into pathogenic, abnormal PrP molecules. They perfectly fit into theoretical predictions of PrP kinetics.

Detection of prions by polymerase chain reaction (PCR) and reverse transcription-PCR is not feasible, as there is no known nucleic acid intimately associated with them. However, the absence of experimental evidence for nucleic acid should not be mistaken as ultimate evidence for the absence of nucleic acid. Serology does not exist since PrP is not recognized as foreign, tolerance prevails, and no immune response is raised that could be exploited to detect persistent prions.

Fortunately, the host seems to contribute to persistence. Individual susceptibility against prions is controlled by genetics, namely, the nucleotide sequence of the host's prion protein gene. Long-term kuru survivors were heterozygous with respect to codon 129; one allele coding for methionine and the other one for valine. To date, all but one vCJD case were homozygous; both alleles coded for methionine.

Despite experimental efforts, no clear-cut results are available on how prions are treated by the invaded cell. To solve these questions, molecular biology, including aforementioned transcriptomics and proteomics combined with prion-infected cell cultures, is required.

Transmission

Interspecies and intraspecies transmission is the hallmark of most, but not all prion diseases and distinguishes them from other amyloidoses such as Alzheimer's disease. Experimentally, animal models for strain typing were key to assess transmission, individual susceptibility, incubation periods, and subclinical stages preceding disease. Transmission of human kuru and CJD and BSE prions into nonhuman primates, such as the chimpanzee, rhesus monkeys, or New World marmoset monkeys, was successful. Guinea pig, rodents, sheep, and cattle (but never rabbits) became versatile recipients in transmission studies. Impressive results on the biology of prions were obtained but at the same time a viral etiology of prion disease was postulated. Since then, small virus particles in the 25°nm range have been claimed to be etiological agents and to be prevalent in the human population. To end this dispute, sophisticated PMCA and cell culture experiments are being applied to confirm either the protein-only hypothesis or a viral etiology, and thus separate the wheat from the chaff when prion hypotheses are to be evaluated. We may be surprised if both factors, virus and prion, are required.

Intraspecies transmission has been shown to be much more effective than interspecies transmission. Inadvertently, transmission from human to human has occurred in neurosurgery and at medical treatment. Experimental sheep-to-sheep transmission has been performed to detect prions in blood and after blood transfusion. Transmission of human vCJD by blood transfusion has also occurred and has motivated blood factor/concentrate suppliers to develop new purification measures for blood or its components. For basic research, experimental prion transmission into hamster, inbred mice, and transgenic mice is indispensable. Prions from mink have been found to impose different clinical phenotypes. As mentioned above, two strains, Hyper and Drowsy, emerged when transmitted into hamster and indicated that prions are heterogeneous. The transmission experiments not only helped define strains but also were pivotal for recognizing that infectivity of prions, the presence of abnormal PrP, and clinical symptoms are three facets of prion infection and disease. It is not mandatory that the three traits coincide in one prion disease. Abnormal PrP is barely detectable in FFI. In GSS, there are clinical symptoms and abnormal PrP is easily detected but transmission is difficult to obtain. Similarly, octarepeat or the respective peptide insertions in PrP result in neurotoxic PrP aggregates but these are noninfectious and do not induce PrPc conversion in a PMCA assay. Inbred mice and genetically altered transgenic mice producing heterologous PrP from other species, denoted as humanized, bovinized, or ovinized, are now routinely used in transmission studies because the susceptibility for prions brought about by the host PrP can be introduced into individual mice. They were most instrumental for assessing prion pathogenicity and infectivity, as well as for detecting novel prion strains.

To summarize, transmission studies of prions allowed insight into traits of strains, such as susceptibility of the host, lesion profiles, persistence and strain splitting, or PrP molecules in pathology. There is ample evidence that PrP molecules encode strains, but how infectivity is gained is yet unsolved. The idea of a transmission barrier rather than a species barrier was suggested when persistence of infectivity and lack of clinical symptoms were recognized in animals. Thus, the assumption that tight species barriers prevent interspecies transmission became disputable. Eventually, these findings led us to identify BSE prions as the causative agent of vCJD in humans and classify BSE as zoonosis.

Contribution of the Host

Susceptibility

Propagation of invading prions seems to be controlled by the host in a manner comparable with conventional pathogens. Either the host is capable of resisting the invasive agent and limits pathogenic consequences or infection inevitably progresses to disease.

Innate and acquired immunity are major defense mechanisms against pathogens. In contrast, prions may not be efficiently recognized by the immune system. Innate immunity and especially acquired immunity fail because the presumed major constituent of prions, the PrP molecules, is encoded in chromosomes of the host and is not recognized as foreign to the organism.

Because prion proteins and their respective genes are conserved in different species, tolerance dominates over the acquired immune response. However, rabbits and goats can be inoculated with PrP, somehow outwitting tolerance to mount a polyclonal antibody response. In contrast, most animals such as laboratory mice are tolerant and respond at best with a marginally acquired immune response with antibodies and a T-helper cell response. Only PrP-deficient mice strongly respond to inoculation and have been the source for widely used monoclonal antibodies.

Tolerance is difficult to break, and when broken therapeutically, could lead to a host-damaging autoimmunity. Although it would have been an elegant strategy to interfere with prion infection at one portal of entry, the lack or inefficiency of an acquired immune response excludes a traditionally active vaccination to confer protection through induction (of mucosal) immunity in Peyer's patches of the intestinal tract.

Experimentally of interest, transgenic animals expressing anti-PrP antibody genes displayed prolonged survival after exposure to prions and monoclonal antibodies added to cells susceptible for prions inhibited or reduced the formation of abnormal PrP. The mechanism of partial protection may reside in binding PrPc and outcompeting cellular factors with affinity to PrP, especially those converting normal PrP eventually into abnormal PrP. In a similar manner, interactions between resident PrP molecules and invading prions are prevented. Neither active immunization nor passive administration of antibodies should be considered as prophylactic or therapeutic measures because physiological functions of the membrane-anchored PrP could be suspended. Breaking tolerance as well as immunostimulatory strategies are double-edged swords. They are essential in basic research but far from becoming an applicable means for treatment of humans or animals. Even small-sized intracellular antibodies attacking PrP in the cell are not appropriate for use in a living organism.

Immunity fails, but the genetic prion PrP gene background of the host appears to control susceptibility and resistance. There is ample evidence that the polymorphism at codon 129 in the PrPc predisposes an individual to disease and influences incubation times. The homozygous genotype methionine/methionine favors the onset of disease in shorter times compared with the heterozygous methionine/valine or homozygous valine/valine combination. Long-term kuru survivors, up to 40 years after exposure, were preferably heterozygous MV, whereas those expressing disease after 20 years were homozygous MM. Genetic data suggested a balancing selection, leading to a high frequency of 55% of heterozygous MV individuals in Papua New Guinea. Most disquieting data are from young vCJD cases. They all belong to the homozygous MM group. The question that arose is what will happen to BSE-infected individuals carrying the MV or the VV allele combination? They may be silent carriers and succumb to disease after decades, comparable with the long-term kuru survivors with MV or VV genotypes. Another polymorphism at codon 219, a lysine instead of a glutamic acid, seemed to render humans resistant to sporadic CJD. This genetically associated resistance resembles a polymorphism in sheep at codon 141, phenylalanine replaced by arginine. The allelic variant with arginine was correlated with a novel, the so-called atypical scrapie phenotype, whereas the allele coding for phenylalanine presented like classical scrapie. Differences between the respective PrP structures and their stabilities could be associated with the phenotype of disease. Breeding of scrapie-resistant sheep with selected PrP genotypes was attempted in hopes of eradication or at least reduction in susceptibility to scrapie in endemic flocks, but this strategy failed. Even worse, it may be dangerous because silent carriers may have been thus generated as has been shown in sheep. Cervid prion diseases are also characterized by a typical amino acid sequence around codon 151, a loop region that may govern crucial PrP interactions upon transmission. The number of known atypical phenotypes of prion diseases in cattle and sheep and the strains underlying them have increased since TSE surveillance in animals has substantially improved.

An intense search for additional genetic markers and quantitative trait loci was initiated after inoculation of mouse-passaged scrapie or BSE into different mouse inbred strains. The limited results of these studies pointed to the H2 haplotype that may confer susceptibility in the species-crossing BSE experiment. Despite many efforts, no definite evidence for particular genes as regulators of susceptibility could be identified.

The PrP gene of the host can control the susceptibility of prion infection, which resembles effects of host genetics on infection susceptibility well known for several pathogens in infection biology. An endogenous gene of the host encodes the PrP, which itself is assumed to be a structural component of the suspected prion, a scenario sometimes observed and understood in microbiology, for example, cholesterol and HIV infectivity. What are the (molecular) biological mechanisms that decide (1) to resist prim infection, (2) just to get attacked, or (3) to progress to disease.

The physiological status of the host, for example, younger individuals with a competent immune system or an individual suffering from an immune suppression, should not be overlooked when susceptibility is discussed. The quality of the lymphoreticular system (LRS) with its cellular and humoral components or the responsiveness of the immune system might modulate prion infection. Folicular dendritic cells (FDC) or an intact complement system support prion infection and are best examples of modifiers. Not the least, sheep infected with an inflammatory Maedi–Visna virus disease made them more susceptible to scrapie.

Spread

When prions have been taken up by an organism, a complex pattern of migration and dissemination emerges. Besides the nervous system with its neuroanatomical pathways, the LRS supports the dissemination of prions. To illustrate this, immunodeficient severe combined immunodeficiency mice progressed more slowly to disease than immunocompetent ones. Immunological knockout mice that do not express complement genes also showed a delayed outbreak of disease and suggested that an intact immune system is necessary for prion infection.

Definite answers regarding nonneuronal transport and specific contributions of cells and tissue are under investigation. Conclusive evidence concerning which molecular processes are responsible is not available. However, detection of PrP molecules has been confirmed in muscle, oral tissue, kidney, and mammary glands; infectivity has yet to be proven with bioassays and PMCA.

Recent studies in 2007 in cattle experimentally infected with BSE favor the neuronal transport of BSE prions as a major if not exclusive pathway for the migration of prions. BSE prions are scarcely seen in the LRS, quite in contrast to scrapie. In sheep, abnormal PrP can be found in tonsils and blood during inapparent stages of prion disease.

The occurrence of blood and blood cells containing abnormal PrP and vCJD prions that have been transmitted by blood transfusion is a sad and alarming reality. For health authorities, the presence of prions in the blood supply became an important issue and hopefully transfusion-transmitted vCJD will not increase beyond the four known cases. The risk of underestimating prion infectivity in health care settings could be compared with the preventable transmissions occurring in the early days of HIV/AIDS in human populations.

The association of PrP molecules, even if they are not prions, with plasminogen has been reported. This is an intriguing finding because plasminogen acts as a virulence factor for the influenza virus, as the neuraminidase of influenza A virus binds and sequesters plasminogen on the cell surface, leading to an enhanced cleavage of the hemagglutinin.

The density or the number of normal PrP on the surface of cells or located intracellularly varies from species to species. Platelets of hamster and humans differ considerably with respect to surface-bound normal PrP. Studies with Syrian hamsters clearly demonstrated how prions, monitored by the presence of abnormal PrP, migrate from the periphery via axons into the central nervous system and eventually arrive in the brain.

How prions are transported from cell to cell is completely unknown. They may be passed on via cell-to-cell contact or be transported by extruded vesicles. Experimentally, migration of prions is reminiscent of herpesvirus type 1 traveling along axons from the ganglia where the virus persists in mucous membranes from which recrudescence begins.

According to recent data, prions in saliva are responsible for an efficient horizontal transmission of CWD leading to apparent disease or, at least, causing a high attack rate observed after pathological examination in local free-ranging or captive cervid populations.

The presence of abnormal and normal PrP molecules in kidney and urine has been substantiated as well as in milk. The search for infectivity is under way.

The host-encoded PrP has convincingly been shown to be key for prion infection because PrP-deficient mice survive and do not progress to disease after experimental infection. Lack of indigenous PrP protected perfectly against disease. PrP deficiency prevents deadly cycles starting with PrPc that might be converted by prions and lead to a new generation of prions to complete the cycle.

In conclusion, PrP is necessary for producing abnormal PrP; it is involved in replication and propagation of prions.

With advanced cell culture systems for prion infection, physiological conditions enabling or coopting a cell to generate prions will likely be found. Biophysical and microscopical techniques such as high-resolution light microscopy and quantum dot approaches are promising tools to image intracellular processes using labeled PrP.

The search for hidden locations of prion replication and carriers or vehicles for transmissible prions has gained high priority. Brain and neuronal tissue are preferred sites for prion replication; yet tonsils, spleen, appendix, tongue, saliva, nose, retina, kidney, muscle, and skin have been found to harbor at least abnormal PrP. To confirm infectivity, transmission experiments, perhaps combined with an initial PMCA, need to be performed.

Diagnostics

Clinical Diagnostics

Clinical diagnostics rely on neurological and physical examination and interviewing relatives of the patient for suspected early signs of disease. The onset of disease is in the past and, unfortunately, no early marker identifying an individual with an evolving prion disease is available. When dementia, ataxia, and myoclonus besides other neurological deficits become visible, they are taken as significant signs of disease, which then progresses within weeks or few months to terminal stages. vCJD patients present with a psychiatric disorder atypical for classical sCJD cases. To date, the clinical parameters are also sufficient for neurologists to differentiate prion disease from other neurodegenerative disorders such as Alzheimer's disease or others. Neurophysiology contributes along with EEG. The so-called typical periodic short-wave EEG activities enable neurologists to diagnose prion disease with high certainty. MRI has developed into an important noninvasive tool for differentiating sporadic CJD from inherited prion disease and, most importantly, acquired vCJD. Similarly, experienced veterinarians can diagnose prion-infected animals according to specific physical and behavioral changes. Cows appear startled, sheep develop scrapie symptoms like scratching, rodents tumble, and cervids with CWD become dehydrated, waste, and die. TSE in different species resemble each other with respect to clinical symptoms. For example, CWD of cervids shares some physical symptoms with human vCJD patients suffering from a dramatic dehydration.

Molecular Diagnostics

The detection of PrP deposits in tissue specimens is routine in postmortem diagnosis with anti-PrP antibodies in IHC, WBs, and paraffin-embedded tissues blot techniques. Abnormal PK-resistant PrP is now regarded as a surrogate marker rather than an etiologically definite marker for prion disease since sometimes abnormal PrP molecules are either not present or seem to escape routine biochemical detection in the limited PK digest or experimental transmission. Brain biopsy specimens can be prepared for IHC to detect abnormal PrP after hydrolytic autoclaving to reduce immunoreactive PrPc and to find histomorphological changes by tissue staining. IHC techniques are permanently improved by fast immunostaining procedures for PrP in automated diagnostic systems and rapid diagnosis (immunohistochemistry) is becoming available. For biochemical typing, abnormal PrP is isolated from brain tissue with sophisticated extraction protocols.

After experimentally limited proteolytic treatment, PrP can be classified according to its tripartite PrP banding pattern that relates molecular weight and quantity of the unglycosylated and the two-glycosylated PrP isoforms described above.

Biochemical banding patterns are then combined with the prion protein genotype of the individual case. For instance, an MV2 case has the biochemical banding pattern of type 2 and its PrP-encoding gene is polymorphic at codon 129; that is one allele coding for methionine and the other for valine. To complete the picture, a second classification protocol was elaborated, resulting in more than five PrP banding patterns that proved to be highly instrumental for identifying strains in transmission studies in selected transgenic mice. In fact, this classification originally confirmed that BSE and vCJD had identical etiologies and shared the PrP type 4 patterns; in other words, the BSE and vCJD diseases represent the action of a single prion strain.

PET blots extended classical IHC to localize pathological PrP in larger areas in brain sections, to assess the loco-lesional distribution and, to a certain degree, the quantity of abnormal PrP. It also allowed for the examination of colocalized cellular proteins suspected to be associated with pathogenicity or relevant for pathological changes.

Although many appropriate anti-PrP monoclonal antibodies are available, only a few of them have been proven to discriminate between the PrPc and the growing number of abnormal PrP isoforms.

Conformation-dependent immunoassays have been developed to elucidate the tendency of PrP molecules to adopt an abnormal PrP conformation. In principle, an appropriate antibody can detect one and the same epitope in one conformer but not in a different closely related PrP one. This technique is excellent for basic science but is error prone in routine testing because of the stringent assay conditions to render an epitope accessible or not.

The detection of PrP by monoclonal antibodies combined with amplification of detector nucleic acid in a subsequent PCR format, called immuno-PCR, is promising but needs validation to meet criteria such as specificity, sensitivity, and intra- and interassay reproducibility. One might expect that the sensitivity of detection of abnormal PrP in the femtogram to low picogram range per ml blood (10e5–10e7 abnormal PrP molecules per ml) would be sufficient for automated assay formats where 10–100 μl volumes of body fluids are to be used. In terms of sensitivity, an immuno-PCR would be about 10e5–10e6-fold more sensitive than standard WB or enzyme-linked immunoabsorbent (ELISA) methods. If appropriately adapted, it might replace highly reliable ELISA or WB test systems in routine diagnostics.

Already validated test systems such as ELISA are continuously and successfully adapted to the needs in human and especially veterinary medicine. To speed up routine diagnostics, the usual proteolytic treatment of source material with PK could be omitted in one BSE/CWD test where PK treatment was replaced by a capture molecule that most efficiently binds abnormal PrP without the loss of specificity and sensitivity.

Considerable efforts are being made to develop a premortem or intravitam diagnostic test kit. To achieve this, new prion test solutions like the ones mentioned have to be fine-tuned and sensitivity considerably improved to detect abnormal PrP in tissue, cells, and body fluids.

Besides monoclonal antibodies as probes for PrP, nanotechnology is expected to broaden the scope of detection of PrP and all its suspected abnormal molecular variants. Candidate molecules to replace monoclonal antibodies might be peptide or nucleic acid aptamers. When provided in biosensor formats, they must be validated to ensure sufficient sensitivity and specificity.

Additional biophysical methods such as spectrophotometry, plasmon resonance, and mass spectrography to detect PrP may soon become available.

Not only in basic research but also in diagnostics, the PMCA was a breakthrough. Trace amounts of brain tissue were shown to induce conformational transition of PrPc into PK-resistant PrP molecules. Most remarkably, prion infectivity could be generated using PMCA product from highly purified cellular hamster PrP, reminiscent of a sporadic birth of prions. PMCA coupled to a bioassay is most likely the method of choice to simultaneously assess the formation of abnormal PrP and infectivity. Similar to PMCA, prion detection has been reported in an amyloid seeding assay.

Besides PrP molecules, other markers have been identified and applied in prion research. The most reliable one is 14-3-3, followed by the tau protein fragments and neuronal-specific enolase. The availability of a combination of marker profiles is basic to clinical diagnostics. However, such markers are valid for certain prion diseases but not for all. 14-3-3 is reliable and reproducibly found in CJD cases but not in FFI or vCJD in which only 50% score positive. None of these markers was qualified for laboratory diagnosis in veterinary medicine.

Easily accessible, non-PrP marker in blood, saliva, milk, and or urine must be identified and evaluated. Unfortunately, one candidate marker, the alpha hemoglobin-stabilizing protein (AHSP) mRNA, cannot be recommended as a discriminatory test, although reduction of the abundance of AHSP mRNA in TSE was initially discussed as a TSE marker. Disappointingly, even sophisticated techniques used in transcriptomics and proteomics have not yet revealed eagerly awaited prion markers for the early detection of disease.

Bioassay

Animal models are inevitable in prion research when infectivity has to be proven and strain characteristics have to be defined. Beyond basic information on the presence of infectivity, physical responses of the host such as the immune system or the internal spread of prions can be investigated. Small rodents such as the hamster, bank vole, or mice strains and especially those transgenic mice designed to mimic a human, bovine, or ovine recipient are available and were successfully used to demonstrate infectivity and strains.

Along with sensitive PMCA amplification, laboratory animals are key to confirm infectivity and perpetuation of strain characteristics. However, cell culture may become an additional tool for research on prion-controlling physiological factors in cells that are important for uptake, in replication and shaping of PrP, and infectious prions. Prion-susceptible permanent cell lines like the neuroblastoma-derived N2a cell line or GT1 cells are prominent examples and are widely used. They were instrumental in the assessment of the role of PrP in physiological intracellular pathways and the initial steps of conformational transition, leading to the accumulation of PK-resistant PrP. Most cell lines were derived from mice, but some were derived from other species such as rabbit. Sublines of currently used cell lines may be established to create another means to titrate prions and to assay replication competence in a given cellular environment. Besides the capacity of cells to multiply abnormal PrP, cell culture experiments may help to find long-sought essential factors that convert PrP into intermediate PrP molecules, turning these into abnormal PrP resistant to proteolysis and into infectious prions. Although a standard scrapie cell assay is expected to become an essential tool for prion research, experiments with animals should not be abandoned whenever the puzzling mutual interactions of prions and the host are of interest.

Conclusion

Disease, infection, transmission, and several PrP variants participate in a complex prion framework with mutual dependencies, and the exact nature of prions remains unknown. Molecular, cellular, and genetic aspects of prion biology continue to provide details as to how this pathogen acts in nature. If the metabolism of normal and mutant PrP is understood, prions may lose their mysterious nature.

Prion disease can be diagnosed on the basis of clinical symptoms and distinguished from other neurodegenerative disorders. Transmission of disease by prions is unique and has been observed both naturally and under experimental conditions. Transfer of disease has been seen in humans after transplantation, blood transfusion, and neurosurgery, which has never been observed in Morbus Alzheimer's or Huntigton's disease. Fortunately, the number of iatrogenic prion cases such as dura mater transplantation was low and measures were successfully taken to prevent human-to-human transmission by transplantation and blood transfusion. Animal models were useful and sensitive, for shedding light on infectious prions. For example, prions from hamster can be transferred into mice but without apparent disease. Later, infectivity was determined, demonstrating that mice were attacked by hamster prions, although they did not proceed to disease. These disturbing results let to the conclusion that infectivity in the absence of disease does exist. What was once interpreted as the species barrier is now called the transmission barrier, which is more appropriate since it prevents the wrong perception that, since disease is absent, prions cannot cross species. Similarly, no abnormal PrP was detected in mice after intracerebral inoculation of BSE, and this subsequently questioned abnormal PrP as the one and only indispensable marker. As to human GSS and its corresponding transgenic mouse models, abnormal PrP can be present at low levels and paired with an efficient transmission capacity. In human FFI, PK-resistant PrP molecules are hardly detectable and transmission is difficult to obtain. Octapeptide insertions into PrP render PrP aggregates neurotoxic but these aggregates seem to be noninfectious because transmission is not observed. Some transgenic mice overproduce PrP but have low levels of PK-resistant PrP molecules; they possess remarkable infectivity.

To summarize, the level of abnormal PrP, clinical disease, and infectivity is dissociated under natural and experimental conditions. Subtle molecular differences control physiological functions, pathogenicity, and infectivity (**Table 1** and **Figure 4**).

A whole scenario of combinations and pitfalls has been found. Low doses of prions may go undetected because not enough exposure is provided to induce clinical symptoms. Low quantities of abnormal PrP may be overlooked because of insufficient sensitivity of the applied test, or abnormal PrP may be hidden in tissues or cells as it was the case for tongue, mucous membrane of the nose, or retina. These observations are of utmost importance as true prion transmission must be assessed; so that testing should not rely only on a biochemical confirmation of abnormal PrP. Prions with an atypical phenotype of disease have been identified, not the least in experimental transmission. In some cases, biochemical typing of abnormal PrP was successful for defining these strains on a molecular basis, showing that certain misfolded PrP molecules can differ from other misfolded ones in objective characteristics responsible for prion strain diversity. A single prion inoculum can diverge into two strains as proven in elaborate transmission experiments in transgenic mice. Two extremes might be anticipated; some PrP molecules contribute to the transmissible infectious agent and eventually disease, whereas others might only be neurotoxic. A schematic representation of individual steps linking physiology, pathogenicity, and infectivity is shown in **Figure 4**.

If mammalian prions are analogous to yeast prion-like proteins, then architecture and conformation of PrP aggregates would be the sole determinants of infectivity like the Sup35 protein aggregates determine a

Table 1 Presence of abnormal PrP, disease, and infectivity. Detection of abnormal PrP, progression to weak pathology or apparent disease, and transmission or infectivity can be dissociated from each other in natural and experimental settings. Scrapie and BSE occur in nature; strain propagation is feasible in certain recipient mice; experimental transmission studies with human octapeptide PrP mutations are an example for an inefficient transmission into mice; a natural transmission barrier may have prevented scrapie from infecting humans

PrP, disease, and infectivity			
Abnormal prion protein[a]	*Disease*	*Transmission + infectivity*[b]	
+	+	+	Scrapie, BSE
−	Pathology[c]	+	Recipient tg mice
+	+	−	Human octapeptide mutations
−	−	Possible	Transmission barrier

[a] Not detectable '−'.
[b] Not observed '−'. Possible: minor residual infectivity.
[c] No clinical symptoms but pathological changes.

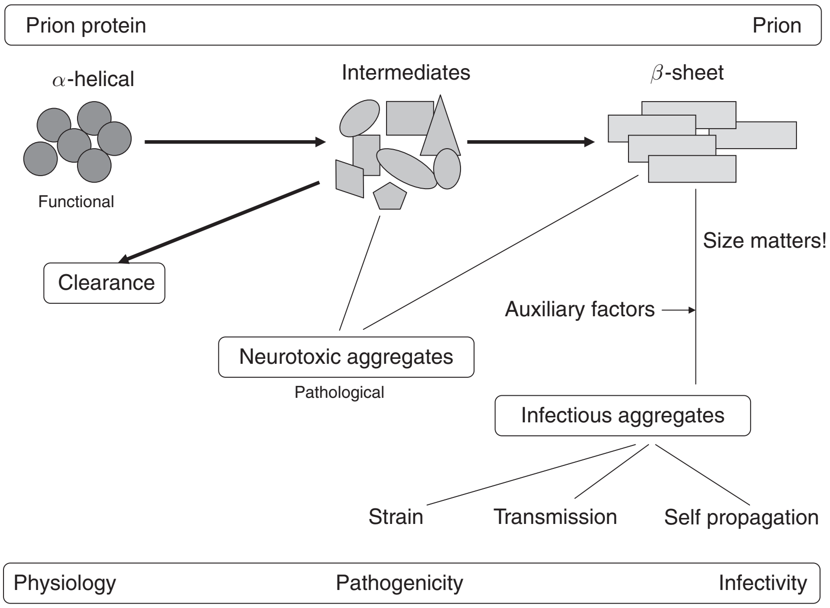

Figure 4 Transformation of cellular PrP into a component of prion disease and prions. Physiology, pathogenicity, and infectivity are associated with a variety of different PrP molecules, which can be integrated into a complex network structure. Intermediates refer to conformationally distinct PrP molecules assumed to become neurotoxic or to initiate prion formation if not cleared in time by intracellular proteolytic degradation.

phenotypical trait in yeast. Such a result would be strong evidence in support of the protein-only hypothesis.

Although experimental progress has been made, prion research is left with many unresolved problems and few firm conclusions, as the constituents necessary for defining the true nature of an authentic prion have not yet been unambiguously identified.

No reliable marker, especially early marker, for prion disease has been found which confirms infection, indicates infectivity or is capable of predicting disease progression at a time when intervention measures would be successful.

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Pseudomonas

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Defining Statement
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Human Pathogens
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Further Reading

Glossary

biofilms Aggregation of microorganisms embedded in an adhesive and protective matrix.

hypersensitive response A plant defense mechanism against pathogenic microorganism.

operon A transcriptional unit encoding one or more genes transcribed from the same promoter to produce a single messenger RNA (mRNA).

opportunistic organism An organism that is generally harmless and becomes pathogenic in an immunocompromised host.

sigma factor A protein that helps the RNA polymerase core enzyme to recognize the promoter at the start of a gene.

transcriptional regulation Mechanisms that regulate the expression of a specific gene or operon.

Abbreviations

γHCH	γ-hexachlorocyclohexane
2-DE	two-dimensional gel electrophoresis
ABC	ATP-binding cassette
AHL	acyl-homoserine lactone
AlgL	alginate lyase
AP	alkaline protease
ASD	aspartate β-semialdehyde dehydrogenase
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CTX	cytotoxin
Dab	2,4-diaminobutyrate
DAP	diaminopimelic acid
ECF	extracytoplasmic function
EF-2	eukaryotic translation factor-2
EPS	exopolysaccharides
ExoS	exoenzyme S
FAS	factor for activating exoenzyme S
HR	hypersensitive response
INA	ice-nucleation-active
IR	inverted repeat sequence
IS	insertion sequence
IVET	*in vivo* expression technology
LPS	lipopolysaccharide
LTTR	LysR-type responsive transcriptional regulators
mRNA	messenger RNA
NRPS	nonribosomal peptide synthetases
OMP	outer-membrane protein
ORF	open reading frame
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyls
PLC	phospholipases C
PLC-H	hemolytic phopholipase C
PMI-GMP	phosphomannose isomerase-guanosine diphosphomannose pyrophosphorylase
PQS	*Pseudomonas* quinolone signal
PVD	pyoverdine
SOD	superoxide dismutase
TCE	trichloroethane
Vfr	Virulence factor regulator

Defining Statement

The genus *Pseudomonas* represents a physiologically and genetically diverse group with a great ecological significance. The article describes this widely investigated and ubiquitous group of bacteria, their pathogenesis in humans, the complex interactions with plants, and their active metabolism in the environment.

Introduction

Pseudomonads are ubiquitous microorganisms, found in all major natural environments and in intimate association with plants and animals. Their wide distribution reflects a remarkable physiological and genetic adaptability. In humans, they are opportunistic pathogens, found in lungs of cystic fibrosis (CF) patients, in people with eye infections, in burn victims, and in AIDS patients. Pathogenicity is due to the secretion of a large number of toxins, which weaken or allow evasion of the host immune system, enabling the bacteria to survive. In plants, they cause disease by producing hypersensitive response (HR), resulting in leaf and root tissue damage. In soils, they detoxify environmentally hazardous compounds, such as aromatic compounds, halogenated derivatives, and various recalcitrant organic residues. The genus *Pseudomonas* includes Gram-negative, non-spore-forming, rod-shaped bacteria, motile by means of one or more polar flagella. They are obligate aerobes, but some species can grow in anaerobic conditions in the presence of nitrate. Catalase positive, they metabolize sugars oxidatively and none is fermentative or photosynthetic. The size of *Pseudomonas* is about 1–5 μm long and about 0.5–1.0 μm wide. The genome sizes vary from 3 to 7 Mb with a substantial genome size polymorphism within each species and a GC content ranging from 58 to 70 mol%. The family Pseudomonadaceae is classified, on the basis of rRNA:DNA hybridization analysis and 16S rRNA sequencing, into five rRNA groups. The current genus of *Pseudomonas* is restricted to the rRNA group I, which belongs to the γ subclass of the Proteobacteria. This genus contains mostly fluorescent *Pseudomonas* spp. as well as few nonfluorescent species. The rRNA group II Pseudomonads belong to the genera *Burkholderia* and *Ralstonia*, organisms of rRNA group III that are now classified in the family Comamonadaceae, while the rRNA groups IV and V form the genera *Brevundimonas* and *Stenotrophomonas*, respectively.

Human Pathogens

Pseudomonas aeruginosa is an opportunistic human pathogen capable of colonizing and infecting virtually any tissue. Since this microorganism is ubiquitous in nature, most human hosts counteract the infectious process effectively via the innate immune system. All clinical cases of *P. aeruginosa* infection are associated with a compromised host defense. The three most common human diseases caused by *P. aeruginosa* are bacteremia in severe burn victims, chronic lung infection in cystic fibrosis (CF) patients, and acute ulcerative keratitis in coal miners, farmers, and users of extended wear contact lenses. *P. aeruginosa* also causes osteomyelitis and urinary tract infections. This pathogen produces many factors that promote adherence to host cells and mucins, damage host tissues, elicit inflammation, and disrupt defense mechanisms. Investigation of these bacterial virulence factors has provided understanding of *P. aeruginosa* pathogenesis at the molecular and cellular level.

Virulence Factors and Pathogenicity

Flagella

Pseudomonas is motile by a single, polar flagellum and exhibits chemotaxis to favorable molecules, such as sugars. The flagella of *P. aeruginosa* have been associated with virulence since nonflagellated mutants do not readily establish infection in animal models and demonstrate reduced invasion of cultured corneal epithelial cells. Strains of *P. aeruginosa* express either an a- or b-type flagellum. This classification is primarily based on the apparent size of the flagellin subunit (encoded by the *fliC* gene) and its antigenicity. The a-type flagellins are heterogeneous and are divided into various subgroups, whereas the b-type flagellins are homogeneous. The a-type *fliC* open reading frame (ORF) varies in length between 1164 and 1185 bp, with the subunit size ranging from 45 to 52 kDa. The b-type *fliC* open reading frame is 1467 bp in length and encodes a 53 kDa size protein. The N- and C-terminal sequences of both these flagellins are nearly identical, whereas the central region is variable. The a-type flagellins undergo glycosylation, whereas the b-type flagellins are phosphorylated at tyrosine residues. Such modifications are unique among the prokaryotic flagella, which are often methylated at the lysine residue. The phosphorylated flagellin protein is believed to serve as a signal for intact flagellin export from cytoplasm to the flagellar assembly apparatus. Expression of flagellar motility requires more than 40 genes controlled by a hierarchical transcriptional regulation organized in four levels. Class I genes are constitutively expressed and include the transcriptional regulator *fleQ* and the alternative sigma factor *fliA* (σ^{28}). FleQ directly or indirectly regulates the expression of the majority of flagellar gene promoters with the exception of *fliA* (*rpoF*). FliA, a member of the alternative sigma factors σ^{28}, regulates transcription of the major flagellin subunit (*fliC*). Transcription of *fleQ* gene is controlled by σ^{70} and is repressed by virulence factor regulator (Vfr). Class II genes include the two-component regulatory system FleSR and require FleQ and RpoN (σ^{54}) for their transcriptional activation. FleR and RpoN regulate expression of class III genes. The anti-sigma factor FlgM binds and inhibits FliA. When FlgM is secreted through the hook-basal body rod structure assembled by class II and III genes, FliA is free to activate expression of class IV genes.

Pili

The type IV pili of *P. aeruginosa* or *N*-methyl-phenylalanine (NMePhe) pili are an important cell-associated virulence factor that plays a crucial role in mediating bacterial adherence and colonization of mucosal surfaces and a flagella-independent method of surface translocation known as twitching motility. Pili also serve as the receptors for bacteriophage. The pili are long polar filaments consisting of homopolymers of a 15–18 kDa protein, called pilin, which is encoded by the *pilA* gene. PilA is first synthesized as a prepilin, which then undergoes processing during its export to produce the final pilin subunit. After cleavage, the leader peptide of the newly generated N-terminus undergoes methylation. There are three other accessory genes, designated *pilBCD*, which are required for the biogenesis of pili. These genes are located adjacent to the pilin structural gene. The *pilD* gene encodes the prepilin peptidase and the methylase that process the prepilin protein. A multimeric outer membrane protein (PilQ) forms gated pores in the outer membrane, through which the pilus is thought to extrude. In *P. aeruginosa*, the *pilQ* gene is located in an operon that includes four other genes (*pilMNOPQ*) also required for pili assembly, twitching motility, and phage sensitivity. The pilin protein is retained in the outer membrane of the cell before its assembly into the intact pilus. Pilin filaments have a diameter of ~5.2 nm, with an average length of 2.5 nm, and the subunits are arranged in a helical array, which forms a hollow cylindrical structure. The pilin C-terminal 12–17 semiconserved amino acid residues are exposed at the tip of the pilus and bind to the asialo-GM1 and asialo-GM2 on epithelial cells.

Transcription of PilA in *P. aeruginosa* is controlled by the RpoN-dependent, two-component regulatory system PilR/PilS. PilS is a transmembrane protein located at the pole of the cell, which responds to a signal that remains to be identified. Twitching mobility in *P. aeruginosa* is also controlled by the sensor–regulator pair FimS/AlgR. Since *fimS* and *algR* mutants lack extracellular pili and FimS and AlgR do not affect PilA expression, these two proteins must be involved in the regulation of some other aspects of the system. In addition, twitching motility is also controlled by Vfr, which may bind cAMP and cGMP, widely utilized by *P. aeruginosa* for global physiological regulation.

Lipopolysaccharide

The lipopolysaccharide (LPS) produced by *P. aeruginosa* is a key factor in virulence, protects the bacterial cells from host defense, and mediates entry into eukaryotic cells. It is a typical Gram-negative bacterial LPS, with a basic lipid A structure formed by an *N*- and *O*-acylated diglucosamine biphosphate backbone that anchors the LPS molecule into the outer leaflet of the bacterial outer membrane. Lipid A binds to a core oligosaccharide region, which can be divided into inner and outer core. The inner core oligosaccharide consists of two 3-deoxy-D-*manno*-oct-2-ulosonic acid residues (Kdo^{I} and Kdo^{II}) and two L-*glycero*-D-*manno*-heptose residues (Hep^{I} and Hep^{II}). The latter can be phosphorylated in three major sites and 7-*O*-carbamoylated on Hep^{II}. LPS phosphorylation, which occurs only in the inner core, is essential for bacterial viability and it is associated with intrinsic resistance to some antibiotics. The outer core contains an *N*-alanylated galactosamine residue, three D-glucose residues, and one L-rhamnose residue. The core structure is linked to the O-antigen, which is responsible for conferring serogroup specificity and diverges in 11 chemical variants (*N*-acyl derivatives of various amino sugars). The LPS outer core binds to cystic fibrosis transmembrane conductance regulator (CFTR) host receptor to mediate bacterial internalization into epithelial cells. This interaction activates NF-κB nuclear translocation; however, a lag in the immune response allows *P. aeruginosa* to establish itself in the lungs. *P. aeruginosa* strains may be either LPS smooth (expressing many long O side chains) or LPS rough (expressing few, short, or no O side chains). Although resistance to serum is conferred by the smooth phenotype, LPS-rough isolates unable to produce O-antigen predominate in CF lungs, which suggest that LPS-mediated serum resistance is not essential for survival of *P. aeruginosa* in CF lungs.

Alginate

In the human pathogen *P. aeruginosa*, alginate is an important virulence factor during infection of human epithelia. Alginate is a linear nonrepetitive copolymer of β-D-mannuronic acid linked to its C5 epimer α-L-guluronic acid via β (1–4) glycosidic bonds. The production of extensive amounts of alginate confers a mucoid phenotype and is associated with the formation of biofilms. In CF patients, *P. aeruginosa* can colonize the lungs and contribute significantly to the disease. Since alginate production confers protection to *P. aeruginosa* from the local immune response and from antibiotics treatment, appearance of mucoid strains in CF lungs leads to a chronic infection and progressive decline in pulmonary function.

Alginate biosynthetic pathway (**Figure 1**) is well characterized and consists of a cluster of 12 genes (*algD*, *8*, *44*, *KEGXLIJFA*). In this cluster, *algA* and *algD* encode enzymes involved in the precursor synthesis. AlgA is the bifunctional enzyme phosphomannose isomerase-guanosine diphosphomannose pyrophosphorylase (PMI-GMP) that acts at the first and third steps of the alginate synthesis. PMI scavenges the fructose-6-phosphate from the metabolic pool and diverts it to the alginate synthetic pathway. The resulting mannose-6-phosphate is directly converted to mannose-1-phosphate by the phosphomannomutase AlgC. This enzyme is also involved in rhamnolipid and LPS synthesis, which reflects its genomic localization outside of the alginate biosynthesis cluster and its own promoter regulation. Finally, mannose-1-phosphate is converted to GDP-mannose by AlgA GMP. GDP-mannose is further oxidized to GDP mannuronic acid by GDP-mannose dehydrogenase encoded by *algD*. Once GDP mannuronic acid is

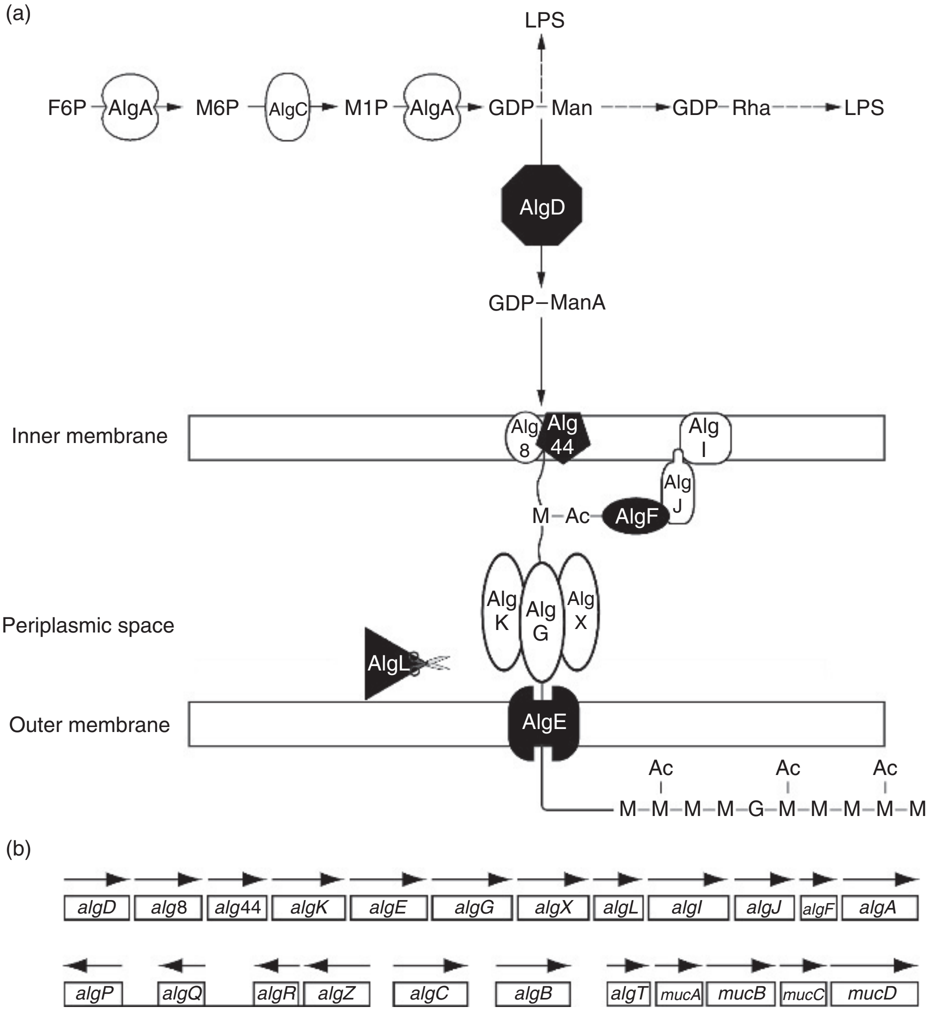
(a)
LPS
F6P
AlgA
M6P
AlgC
M1P
AlgA
GDP–Man
GDP–Rha
LPS
AlgD
GDP–ManA
Inner membrane
Alg 8
Alg 44
Alg I
Alg J
M–Ac
AlgF
Periplasmic space
Alg K
Alg G
Alg X
AlgL
Outer membrane
AlgE
Ac
Ac
Ac
M–M–M–M–G–M–M–M–M–M
(b)
algD
alg8
alg44
algK
algE
algG
algX
algL
algI
algJ
algF
algA
algP
algQ
algR
algZ
algC
algB
algT
mucA
mucB
mucC
mucD

Figure 1 (a) *Pseudomonas aeruginosa* alginate biosynthesis. Fructose-6-phosphate (F6P), obtained from the metabolic pool, is converted to GDP-mannuronic acid (GDP-ManA), which provides mannuronate residues (M) for polymerization. Occasionally, guluronate residues (G) are incorporated via epimerization of mannuronate residues by the AlgG protein. Mannuronic acid residues of bacterial alginate are partially *O*-acetylated by the membrane complex formed by AlgF, AlgJ, and AlgI proteins. The dashed arrows indicate enzymatic steps leading to lipopolysaccharide (LPS) synthesis. Abbreviation used: M6P, mannose-6-phosphate; M1P, mannose-1-phosphate; GDP-man, GDP-mannose; GDP-rha, GDP-rhamnose; and Ac, *O*-acetyl groups. (b) The organization of the alginate gene clusters. The alginate genes are clustered at three locations in the *P. aeruginosa* chromosome. The arrows above the genes represent the direction of transcription. Reproduced from May T and Chakrabarty AM (1994) *Pseudomonas aeruginosa*: Genes and enzymes of alginate synthesis. *Trends in Microbiology* 2: 151–157.

synthesized in the cytosol, the membrane-associated *alg8* and *alg44* gene products form an alginate-polymerizing complex that catalyze alginate polymerization and transfer through the inner membrane. After polymerization, some of the mannuronate residues are epimerized to guluronate residues by a C-5-epimerase (AlgG). This polymer is randomly acetylated by the membrane complex formed by *algF*, *algJ*, and *algI* gene products.

AlgG is supposed to interact with AlgK and AlgX to form a scaffold in the periplasm that protects the growing alginate polymer from degradation by the alginate lyase (AlgL). Alginate lyase is important for the bacteria in detaching from the cell surfaces to spread to new habitats and in generating short oligosaccharides, which are used as primers for new alginate chains. After acetylation, the alginate polymer is transported out of the cell through the outer membrane porin called AlgE.

The regulation of alginate biosynthesis is based on a complex transcriptional control and the extracytoplasmic function (ECF) sigma factor σ^{22} is at the apex of a hierarchy of regulators that control genes for alginate production. σ^{22} encoded by *algT* (also known as *algU*) is required for the activation of the *algD* promoter, which controls the alginate biosynthetic operon (*algD-algA*). AlgT induces expression of other regulators binding the *algD* promoter, including AlgB, AlgR, and AlgZ. AlgR also regulates alginate production through *algC* by binding to its promoter. Moreover, AlgT positively regulates its own transcription by binding to the promoter of the *algT-mucABCD* operon.

The *mucA* and *mucB* gene products inhibit σ^{22} activity by sequestering it from RNA polymerase. Mutations in these two genes cause deregulation of AlgT and conversion to mucoidy as observed in strains of *P. aeruginosa* infecting CF lungs. MucD (AlgW) is a protease under the control of the σ^{22}-independent promoter located in *mucC* and it is supposed to destabilize the MucA–σ^{22} interaction. Synthesis of alginate is a response to several environmental stimuli, such as osmolarity, nitrogen limitation, phosphate limitation, dehydration or stress conditions, and antibiotics.

Siderophores

Siderophores are iron(III) chelators secreted by aerobic bacteria in response to iron limitation. Iron is essential for their metabolism and it is frequently not readily available to bacteria because it is mainly in an insoluble ferric form or host species actively withhold iron from the infecting bacteria. Consequently, siderophore production is crucial for the success of bacteria and results in more virulent infections. After binding iron from the environment, siderophores are selectively recognized by high-affinity receptors on the bacterial cell surface. Pyoverdines (PVDs) are complex siderophores produced by fluorescent *Pseudomonas*, although some species can also synthesize additional siderophores such as pyochelin or quinolobactin, or can acquire iron bound to exogenous chelators, including heterologous siderophores.

PVDs consist of a conserved fluorescent dihydroxyquinoline (chromophore) bound to a side chain, generally a dicarboxylic acid or an amide, and to a strain-specific peptide of 6–12 amino acids that is essential for recognition from the corresponding receptor. More than 50 PVD structures have been elucidated to date and this structural diversity and the receptor specificity have formed the basis for a *Pseudomonas* spp. typing system called siderotyping. Both the chromophores and the peptide chains are synthesized by nonribosomal peptide synthetases (NRPSs) that enable peptide bond formation between amino acids that cannot be incorporated through ribosomal synthesis. These large enzymes have a modular organization, with each module (about 1000 amino acids) catalyzing the incorporation of one amino acid into a peptide. Each module or cassette typically contains activation, thiolation, and condensation protein domains, which charge, bind, and connect a specific amino acid to a growing peptide. The number and order of the modules usually corresponds to the amino acid number and sequence in the peptide product. The synthesis of the peptide backbone precedes chromophore maturation, but events leading to the maturation of the PVD chromophore have not yet been fully characterized.

The pyoverdine-related genes and their chromosomal organization are best known for *P. aeruginosa* PAO1. This strain has four genes, *pvdL*, *pvdI*, *pvdJ*, and *pvdD*, which encode peptide synthetase enzymes and determine the order of amino acids in the PVD peptide. PvdH is the enzyme that generates 2,4-diaminobutyrate (Dab) for incorporation into the PVD precursor peptide, and PvdA and PvdF are the enzymes that catalyze the synthesis of fOHOrn. Other biosynthetic genes (*pvcABCD*) are involved in the chromophore formation. PvdQ is a periplasmic acylase essential for PVD production and release in the milieu, which suggests that an acyl group retains the PVD precursor at the membrane to facilitate the interaction with the membrane-associated biosynthetic machinery. The hypothesis is that a PVD precursor is synthesized in the cytoplasm, transported to the periplasm, perhaps through the ABC transporter *PvdE*, and secreted out of the cell by a not yet clarified mechanism.

The *pvd* region was identified as the most divergent alignable locus in the genome of several *P. aeruginosa* strains, reflecting the strain-specific diversity of PVD structure. In this region, the outer membrane pyoverdine receptor *fpvA* is the most divergent gene, which is not generated by intratype recombination and shows evidence of positive selection. Adjacent to *fpvA*, the genes *pvdE*, *pvdI*, *pvdJ*, and *pvdD* are also highly divergent and probably coevolve to maintain mutual specificity.

The first regulator of siderophores synthesis and uptake to be described was the repressor protein Fur (ferric uptake regulator), which requires ferrous iron for DNA binding. The Fur–iron complexes bind the promoters of iron uptake genes, so the transcription of these genes is shut off in iron-repleted cells. In iron-starved cells the apo-Fur cannot bind DNA and so iron uptake genes are transcribed. However, an additional level of regulation involves sigma factors and enables bacteria to respond to the presence of specific siderophores in the environment as well as to levels of intracellular iron. The ECF sigma factor PvdS in *P. aeruginosa* binds to an iron starvation (IS) consensus sequence that is found upstream of several genes or gene clusters involved in PVD synthesis, regulation, and transport. This sequence is also present in the promoters of genes regulated by the PvdS homologues PfrI in *Pseudomonas putida*, PbrA in *Pseudomonas fluorescens*, and PsbS in *Pseudomonas* B10.

PVDs have several important roles in Pseudomonas biology, including production of virulence factors and development of biofilms. Pyoverdine controls production of two secreted virulence factors of *P. aeruginosa*, exotoxin A and PrpL protease, through a signaling system involving the receptor FpvA, the antisigma factor FpvR, and the ECF sigma factor PvdS.

Other virulence Factors Secreted by *P. aeruginosa*

In addition to LPS, alginate, and siderophores, *P. aeruginosa* employs a multitude of extracellular virulence factors to successfully cause diverse acute infections or to persist as a chronic colonizer. *P. aeruginosa* proteases are crucial for many aspects of pathogenesis on mucosa. They destroy the structural integrity of the host cells by degrading the structural proteins of the extracellular matrix, such as elastin, laminin, and collagen. Moreover, they degrade components of the complement system, human immunoglobulins and serum alpha proteins. At least three proteases have been characterized: an elastase (PE), a general protease (LasA), and one alkaline protease (AP). The PE is a zinc-protease, encoded as a pre-pro protein of 53 kDa by the *lasB* gene. This protein is translocated to the periplasm, where it undergoes autoproteolysis to generate the 18 kDa and 33 kDa peptides, which interact noncovalently. The active 33 kDa elastase is secreted to the extracellular environment by the type II secretion system. The *lasB* gene is regulated in a quorum sensing-dependent manner. The LasA protease can cleave elastin, hydrolyze β-casein, and lyse Staphylococci. The *lasA* gene encodes a 41 kDa precursor that is subsequently cleaved to produce the 22 kDa active protein. This protease is active over a broad pH range and is considered a major virulence factor. *P. aeruginosa* AP is encoded by the *aprA* gene. Secretion of this protease requires three accessory proteins, AprD, AprE, and AprF. These three proteins form a complex that spans the periplasm to allow for secretion of the protease to the external environment in a single step.

Exotoxin A (encoded by *toxA*) is the most toxic protein produced by *Pseudomonas*. In a manner akin to that of diphtheria toxin, this protein catalyzes ADP ribosylation of the eukaryotic translation factor-2 (EF-2) to form ADP-ribosyl-EF-2, which results in inhibition of host cell protein synthesis. The *toxA* gene is expressed at higher levels when the environmental iron levels are low and is governed by the iron-responsive Fur protein. Another extracellular toxin is the acidic cytotoxin (CTX) of 25 kDa that forms pores in the lipid layers of leukocytes. CTX is encoded by a lysogenic phage that is 35 kb in size and is integrated at the *attP* site on the chromosome. This toxin is localized in the bacterial periplasmic space in an inactive or weakly active form, but is converted to the active form by proteases.

Exoenzyme S (ExoS) is a 43 kDa protein encoded by *exoS* and is similar to YopE of *Yersinia enterocolitica.* It has ADP phosphorylation activity toward eukaryotic proteins such as Vimentin, H-Ras, and K-Ras types of GTP-binding proteins. The ADP ribosylation step requires a eukaryotic protein called FAS (factor for activating exoenzyme S). The *exoT* gene encodes another FAS-dependent ADP-ribosylating enzyme called ExoT. Both ExoS and ExoT possess cytotoxic activity and are secreted by the type III secretion system upon host cell contact. Both the *exoS* and *exoT* genes are positively regulated by the transcriptional regulator ExsA.

P. aeruginosa host invasiveness and ability to cause tissue damage is linked to its complex extracellular lipolytic system including at least two phospholipases C (PLC), one outer membrane-bound esterase and one secreted lipase. The 26 kDa secreted lipase is active against a broad range of triglycerides with fatty acyl chain lengths varying from C6 to C8 and it is stereoselective for *sn*-1 of the triglyceride. It is encoded as a 29 kDa precursor by the *lipA* gene. The pre-lipase is secreted by the Sec-dependent secretion pathway into the periplasm, where it interacts with the membrane-bound lipase-specific foldase Lif. While oxidoreductases catalyze the disulfide bond formation of LipA, Lif assists the correct conformational folding of LipA. The resulting mature 26 kDa form of the lipase is then secreted to the external environment via the type II secretion pathway. The lipase operon *lipAlif* is under the control of the σ^{54}-dependent promoter regulated by the two-component system LipR/LipQ. The transcription of the *lipRQ* operon is activated by the quorum sensing activator RhlR. The sensor kinase LipQ may also be activated by so far unknown environmental stimuli or by periplasmic signals including misfolded or nonsecretable enzymes. A 55 kDa esterase tightly bound to the outer membrane has been identified in lipase-negative deletion mutants of *P. aeruginosa.* It preferentially hydrolyzes long-chain acyl thioesters or oxyesters. In addition, two types of phospholipase C have been characterized in *P. aeruginosa*, the hemolytic phopholipase C (PLC-H) and the nonhemolytic phopholipase C (PLC-N). PLC-H is a 78 kDa heat-labile protein that hydrolyzes phosphatidylcholine in erythrocyte membranes; it is also active on sphingomyelin. Its production is regulated by the *plc* operon including the structural gene *plcH* and *plcR1* and *plcR2*, the last two encoding proteins that modify PLC-H after translation. PLC-N is a 73 kDa protein that acts on phosphatidylcholine and phosphatidylserine. The location of the *plcN* gene in the chromosome is quite distant from that of PLC-H. The synthesis of both PLC-H and PLC-N is stimulated under low-phosphate and aerobic conditions. The synergistic effect of lipases and PLC leads to hydrolysis of the lung surfactant dipalmitoylphosphatidylcholine, which causes tissue damage and inflammation. Release of choline leads to the accumulation of betaine, which acts as an osmoprotectant, thereby enhancing bacterial survival within host tissues.

P. aeruginosa produces glycolipid biosurfactants, called rhamnolipids, which can, at high concentrations, disrupt intercellular junctions in epithelia. Rhamnolipids are also implicated in the maintenance of fluid channels in mature biofilms. The production of rhamnolipids is under quorum sensing control.

Bacteriocins, called pyocins, are secreted into the environment and play a significant role in the ecological dominance of this species by promoting the lysis of competing bacteria. However, production of pyocins is more frequent in clinical situations than in the environment, which suggests a role of these molecules in human diseases. Three groups of pyocins, R, F, and S, have been characterized. The R and F types are rod-like particles resembling a bacteriophage tail. The S-type pyocins are the most abundant low-molecular-weight pyocins, including S1, S2, S3, and AP41. S1 and S2 pyocins are able to inhibit phospholipid synthesis of target bacteria under iron-limited conditions, whereas AP41 possesses endonuclease activity and induces the synthesis of other pyocins (R2 and S2) as well as induction of phage. The mode of action of S3 remains to be clarified. Each S-type pyocin consists of two proteins associated in a complex. Only the large protein shows bactericidal activity, while the small protein protects the host cell from the killing activity of the bigger component. Pyocins structural genes are chromosome-located and their transcription is activated by PrtN. This positive regulator binds to the conserved P box sequences located 60–100 bp upstream of RBS of pyocins genes. Pyocin synthesis is induced by DNA damaging agents, which increase expression of the DNA repair protein RecA that cleaves the repressor protein PtrR and liberates the expression of activator PrtN. Pyocin typing has been an epidemiological tool to discriminate one *P. aeruginosa* strain from another to follow its propagation in nosocomial infections.

Two types of sugar-binding proteins called lectins are among the set of virulence factors produced by *P. aeruginosa.* PA-1L is a lectin specific to D-galactose and its derivatives; PA-11L shows specificity for L-fucose and D-mannose. These lectins help the bacteria to adhere to host cells and stimulate an inflammatory response by inducing the host immune system to produce cytokines such as IL-1 and IL-6.

Fluorescent *Pseudomonas*, including *P. aeruginosa*, produce and secrete phenazines, which are heterocyclic, redox-active compounds toxic to competing bacteria. The most studied phenazines are pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxylic acid. There is evidence to suggest that phenazines can penetrate biological membranes and alter cytokine production and signaling pathways in cultured airway epithelia. Also, a recent report indicates that pyocyanin is capable of regulating the expression of a set of *P. aeruginosa* genes.

P. aeruginosa Infections in Cystic Fibrosis

CF is an autosomal recessive disorder caused by mutations in the CFTR (CF transmembrane conductance regulator) gene. These mutations result in defective chloride transport across epithelia. The airways of individuals with CF are susceptible to recurrent bacterial infections, and the opportunistic pathogen *P. aeruginosa* can chronically colonize the lungs of CF patients despite aggressive antibiotic treatment. It has been hypothesized that *P. aeruginosa* in the CF lung may exist as biofilms wherein bacteria are organized in a self-produced polymeric matrix. The biofilm mode of lifestyle may be responsible for the antibiotic resistance of the bacteria in CF. Chronic persistence of *P. aeruginosa* in CF is often accompanied by bacterial adaptations that involve the repression of certain virulence factors. For example, unlike environmental strains, isolates from chronic CF infections often show a repression of flagellin and pilin expression and repression of the type III secretion system. Sustained repression may lead to mutations that result in a permanent adaptation to the unique environmental niche of the airways.

P. aeruginosa Virulence in Burn Infections and Keratitis

P. aeruginosa contributes to burn wound infection with many virulence factors. Pili and flagella are responsible for its adherence and particularly for its dissemination throughout the whole organism. Dissemination is also dependent upon production of elastases and proteases, which destroy the host physical and immune barriers that normally might inhibit the spread of the infection. Disruption of *lasR* regulatory gene that controls the QS response blocks the dissemination from the initial site of infection. QS is responsible for the regulation of several virulence factors, including elastases and biofilm formation. However, the block of dissemination caused by *lasR* knockout is not caused specifically by inactivation of elastases, suggesting the critical role of other virulence factors in bacterial dissemination.

The cell-associated and secreted virulence factors that contribute to the pathogenesis of wound infections are responsible for invasion and destruction of the cornea in microbial keratitis. Another factor that contributes to this destructive process is a continuous recruitment of polymorphonuclear neutrophils in the corneal tissue triggered by the recognition of *P. aeruginosa* flagella or LPS by epithelial cells toll-like receptors. In addition to its role in this inflammatory response, *P. aeruginosa* LPS is a ligand for the epithelial CFTR receptor. The interaction LPS–CFTR receptor causes internalization of *Pseudomonas* by the corneal basal epithelium where bacteria replicate.

Some Unique Behaviors Exhibited by *Pseudomonas*

Antibiotic resistance

P. aeruginosa exhibits an intrinsic resistance to antibiotics and has a demonstrated ability to acquire genes encoding resistance determinants. This resistance is mainly due to production of β-lactamases, diminished membrane permeability to antibiotics, or upregulation of efflux pumps. Unfortunately, multiple mechanisms of resistance can accumulate in some strains leading to the development of multiply resistant strains. *P. aeruginosa* major defense mechanism against the β-lactam group of antibiotics (penicillins, cephalosporins, monobactams, and carbapenems) is the production of a variety of β-lactamases. Despite these lactamases, imipenem resistance in *P. aeruginosa* is commonly generated via a mutational loss of a 54 kDa outer-membrane protein (OMP), usually known as OprD (or the D2 porin). Furthermore, structural modifications of the outer membrane, such as absence of 2-hydroxylaurate, presence of 4-aminoarabinose, and increase of palmitate, are responsible for resistance to colistin. However, efflux pump systems are the major cause of multidrug resistance and the most commonly observed pump system in *P. aeruginosa* is the MexAB-OprM, which consists of a pump (MexB) connected to the outer membrane by the linker lipoprotein MexA and the exit portal OprM. *P. aeruginosa* uses this upregulated MexAB system to export quinolones, penicillines, and cephalosporins, while the upregulated MexXY-OprM efflux pump is responsible for aminoglycoside resistance. Quinolone resistance is attributable not only to efflux pumps but also to mutations of *gyrA* and *parC* genes encoding topoisomerases II and IV, respectively.

Few treatment options are available for multidrug-resistant *P. aeruginosa*: cefepime and amikacin might be active against some strains, otherwise polymyxins remain the most effective agent alone or in combination with one or more of the following: a carbapenem, aminoglycoside, quinolone, or β-lactam.

Response to oxidative stress

Pseudomonas respond to both endogenous (aerobic growth) or exogenous (anaerobic or in macrophage) oxidative stress (superoxide anion O_2^-) by producing iron- or manganese-containing superoxide dismutase (SOD) metalloenzymes. The dismutases catalyze the disproportionation of $O_2{}^-$ to H_2O_2 and O_2. The organism also possesses catalases that remove the toxic H_2O_2 product. The SOD A (encoded by *sodA*) is a 23 kDa dimer, which uses manganese as a cofactor, and the SOD B (encoded by *sodB*) is iron-dependent and also functions as a dimer. Mucoid strains have been observed to possess higher manganese SOD activity.

Cell–cell signaling in P. aeruginosa

Bacteria employ a mechanism of cell–cell signaling called quorum sensing to coordinately regulate gene expression in response to changes in population density. The quorum sensing circuit was first characterized in the marine bacterium *Vibrio fischeri* and includes genes encoding the signal synthase, LuxI, and the signal receptor, LuxR. *P. aeruginosa* utilizes two homologous acyl-homoserine lactone (AHL) quorum sensing systems to regulate the expression of a large number of genes including virulence factor genes and genes involved in biofilm development. These two systems are the LasR-LasI and RhlR-RhlI systems. LasR is a transcriptional activator that responds to the product of the LasI synthase, *N*-3-(oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL). At sufficient environmental concentrations of this AHL signal, a number of genes are activated, including *rhlR*, which codes for the *N*-butyrylhomoserine lactone (C_4-HSL) receptor, and *rhlI*, which codes for the C_4-HSL signal generator. RhlR and C_4-HSL activate many other genes. Transcriptome analyses studies indicate that *P. aeruginosa* regulates over 300 genes in a quorum-dependent manner. The set of quorum-controlled genes includes those coding for the virulence factors pyocyanin, hydrogen cyanide, elastase, and AP. Analysis of the *P. aeruginosa* genome revealed a gene coding for a homologue of LasR and RhlR but no additional genes coding for LasI and RhlI homologues. This 'orphan receptor' termed QscR responds to the product of the LasI synthase, $3OC_{12}$-HSL, and controls a set of genes that partially overlap the Las-Rhl quorum regulon. As might be expected in a versatile bacterium inhabiting diverse habitats, the elements of the two quorum sensing systems are controlled by other factors.

In addition to the AHL quorum sensing systems, *P. aeruginosa* utilizes another low-molecular-weight hydrophobic molecule, 2-heptyl-3-hydroxy-4-quinolone, referred to as the *Pseudomonas* quinolone signal (PQS) for intercellular communication. This signal functions as a coinducer for the transcriptional regulator PqsR to activate the expression of multiple virulence genes and its own synthesis. PQS is one of several quinolones and quinolines made by *P. aeruginosa*. Remarkably, PQS, released in membrane vesicles, which, in addition to signaling to *P. aeruginosa* cells, shows potent antibacterial activity against the Gram-positive bacterium *Staphylococcus aureus*.

Secretion System in *Pseudomonas*

Pseudomonads rely on several pathways for the secretion of toxins, hydrolytic enzymes, and proteins important for virulence. These include the type I, II, III, IV, and VI pathways. The type I secretion (ABC exporter) system utilizes three proteins, an ATP-binding cassette (ABC) protein, a membrane fusion protein, and an outer membrane protein for the secretion of virulence factors to the extracellular environment. The type II secretion system functions in conjunction with the Sec or Tat transport systems for protein transport across the inner membrane. This system allows the transport of proteins across both the inner and outer membranes in a single step. In *P. aeruginosa*, the Xcp and the Hxc type II secretion systems allow for the extracellular release of elastase, lipases, exotoxin A, and alkaline phosphatase. The proteins of the Xcp secretion system share several features with proteins involved in the assembly of type IV pili that are required for twitching motility, adherence to host cells, and biofilm formation. The type III secretion system allows the direct injection of toxins into host cells via a secretion complex that spans the bacterial cell envelope and penetrates the host cell membrane. Recent studies indicate the presence of a type VI secretion system in *P. aeruginosa* and suggest a role for it in the direct injection of virulence factors into host cells.

Plant Pathogens

Several *Pseudomonas* species cause plant diseases in some of the most commercially important crops. Among these species, *Pseudomonas syringae* is the most widespread and best studied. *P. syringae* is taxonomically subdivided into more than 50 pathovars (pathological variants), which are typically distinguished by plant host range. Although the *P. syringae* species as a whole causes plant diseases on a multitude of hosts, individual *P. syringae* pathovars typically have a limited host range of one to a few plant species. Symptoms of diseases caused by *P. syringae* include leaf spots, fruit spots, and cankers on woody hosts. *P. syringae* diseases are currently mainly managed through the use of bactericides and through host resistance in certain crops. However, the continued expansion of understanding of host–pathogen interactions is expected to foster the utilization of host resistance in many more disease pathosystems.

P. syringae pathogens utilize an impressive array of virulence factors such as effectors, toxins, and phytohormones to incite disease symptoms. The most important pathogenicity determinant is the presence of a type III secretion system, which is encoded by genes present in the *hrp* pathogenicity island. *hrp* (hypersensitive response (HR) and pathogenicity) genes were discovered in the early 1980s as genes affecting the ability of strains to elicit a HR in the reporter tobacco plant. The HR was later found to be a plant disease resistance response initiated after the intracellular recognition of pathogen effector proteins delivered via type III secretion. Like most other bacterial plant pathogens, *P. syringae* encodes a type III secretion system that consists of a Hrp pilus, a long syringe-like structure that must traverse the plant cell wall and that enables delivery of effector proteins directly into plant host cells. Development of microarrays and subsequent bioinformatic and functional genomic analyses have enabled the identification of the *hrp* regulon and the complete effector repertoire of a single *P. syringae* strain. These studies have established that active effector genes in *P. syringae* are expressed by the HrpL alternative sigma factor, which recognizes the 'hrp box' motif in the promoter of the *hrp* operons and effector genes. *hrp* induction requires *hrp*S and *hrp*R. Since *hrpL* is under the control of a σ^{54}-dependent promoter in RpoN-dependent manner, it has been hypothesized that the heterodimer HrpR–HrpS interacts with RpoN and promotes *hrpL* transcription. *HrpRS* transcription is positively regulated by the GacS/GacA system, a highly conserved bacterial regulatory system that controls the expression of many cellular functions. However, it is not clear which signal is sensed by GacS and how GacA regulates *hrpRS* and *rpoN* transcription. In addition, HrpA, a major component of the type III pilus, acts as a positive regulator on *hrpRS* transcription by a mechanism that remains to be clarified. In this complex regulatory network, a negative control is played by the ATP-dependent Lon protease that degrades the HrpR protein and by the HrpV protein, which acts as an anti-activator of HrpS.

Exopolysaccharides (EPS) and toxins allow *P. syringae* to cope with environmental condition and host response. *P. syringae* produces at least two EPS, levan, a β-(2,6) polyfructan, and alginate. The latter is widely produced during plant infection and is responsible for lesions having a typical water-soaked appearance. EPS chelate heavy metals, such as copper, increasing tolerance to toxic pesticides and resistance to dessication. Like *P. aeruginosa*, most strains of *P. syringae* are normally nonmucoid and alginate production is activated by stress stimuli. The biosynthesis of alginate in *P. syringae* is similar to that described for *P. aeruginosa* and the arrangement of the alginate structural genes is conserved, although in *P. syringae muc D* transcription is not dependent on AlgT; *muc D* has its own promoter and it is not cotranscribed with the *algT–muc* operon. Interestingly, *mucC* has not been found in *P. syringae.*

P. syringae produces several toxins different in structure and origin, which are not required for its pathogenicity but enhance *P. syringae* virulence, causing plant lesions and facilitating bacterial invasion and spreading in the plant. Syringomycin and syringopeptins are cyclic lipodepsinonapeptide phytotoxins secreted by *P. syringae* pv. siringae. Syringomycin targets the plasma membrane of the host cells, and disrupts the ion transport and membrane electrical potential, causing cytolysis. This necrotic toxin is synthesized by NRPSs. The genes dedicated to syringomycin biosynthesis (*syrB1*, *syrB2*, *syrC*, and *syrE*), secretion (*syrD*), and regulation (*syrP*) are organized in a gene cluster (*syr*) on the chromosome of *P. syringae* pv. siringae. Syringomycin production is modulated by both nutritional factors and plant signal molecules, such as phenolic glycosides, although the mechanism responsible for transduction of these signals to the *syr* transcriptional apparatus is still under investigation. Syringopeptins represent another class of lipodepsipeptide phytotoxins synthesized by a different set of biosynthetic genes organized in the *syp* gene cluster. *Syp-syr* genes are coregulated and respond to the same environmental stimuli. Another interesting phytotoxin is the polyketide coronatine, which structurally mimics the phytohormone jasmonic acid. This hormone regulates fruit abscission and senescence in higher plants. Coronatine causes chlorosis, induces hypertrophy, and inhibits root elongation. Recent studies suggest that coronatine might enable *P. syringae* to colonize the leaf interior by counteracting the plant defensive closure of the stomata. In addition to phytotoxins, *Pseudomonas* secretes amidases and pectate lyase, which play an important role in *Pseudomonas* infection of plants and fruits and destroys the appearance and quality and commercial value of the produce.

Many pathovars of *P. syringae* also possess an epiphytic phase as part of their life cycle. Growth as an epiphyte on plant leaf surfaces enables the buildup of population size, which seems to be a requirement for pathogenesis. The leaf surface or phyllosphere is a habitat that is exposed to various environmental stress factors, and desiccation and exposure to ultraviolet radiation may be the most important. Strategies of tolerance or avoidance of stress are two possible fitness strategies of foliar pathogens and colonists in response to environmental stress. In *P. syringae*, traits that facilitate survival in response to environmental stress such as motility, tolerance to ultraviolet radiation, or EPS production are important to epiphytic population size. This organism commonly forms aggregates on leaf surfaces; these aggregates may form at nonrandom sites of carbon source deposition on leaves. Aggregates are important for phyllosphere survival and appear important in population increases, resulting in ingress into leaves and eventual pathogenesis. Biological control strategies aimed at reducing epiphytic populations have proven successful in some pathosystems.

The ability of some *P. syringae* strains to serve as ice nuclei and nucleate ice formation may be an important factor in plant wounding and ingress of bacteria into plant tissues. In the absence of an ice nucleus, purified water can supercool to temperatures far below 0 °C; ice-nucleation-active (INA) *P. syringae* cells can catalyze ice formation at relatively warm temperatures of −2 to −5 °C. Ice formed in susceptible plant tissues can rapidly propagate; following thawing, this injured tissue is then susceptible to infection.

Most *P. syringae* strains contain extrachromosomal plasmids, and many of these are related, belonging to the pPT23A plasmid family. *P. syringae* plasmids encode various traits beneficial to epiphytic growth and/or virulence. Examples include type III effectors, chemotaxis receptors, ultraviolet radiation tolerance genes, toxin biosynthesis gene clusters, and genes encoding indole acetic acid biosynthesis. Many *P. syringae* plasmids are also conjugative. The importance of gene transfer in the evolution of *P. syringae* is indicated by the evolutionary relationships of various pathovar strains. The observation that closely related strains can belong to different pathovars with distinct host ranges implies the transfer of effectors conferring host range alterations between strains.

Genome sequencing has provided the raw material for determining answers to various questions concerning pathogenesis and host range in *P. syringae.* Since 2003, the genome sequences of *P. syringae* pv. tomato DC3000, *P. syringae* pv. phaseolicola 1448A, and *P. syringae* pv. syringae B728a have been published. In addition, genome sequences from plant-associated *P. fluorescens* strains and *P. aeruginosa* PA14 have also been published. Comparative genomics will facilitate the understanding of host range determinants and the understanding of pathogenesis in this organism. Such studies will hopefully lead to novel disease control methods in plants either through host resistance or through targeting important virulence determinants in the pathogen.

Environmental Aspects of *Pseudomonas*

Degradation of Organic Compounds

Pseudomonas are widespread in many natural environments, where they carry out a variety of biochemical conversions and mineralize organic carbon. They metabolize a large number of natural organic compounds, including aromatic hydrocarbons and their derivatives. The enzymes involved in the degradation of these compounds are generally plasmid-encoded and have low substrate specificity. These two features allow rapid evolution of new metabolic pathways for the degradation of toxic synthetic compounds (xenobiotics), such as highly chlorinated aromatics used as pesticides, herbicides, or by-products released into the environment by industrial processes.

Pseudomonas *degrade chlorinated aromatic hydrocarbons*

Pseudomonas can utilize a wide variety of chlorinated aromatics as sole source of carbon and energy. The processes involved in the degradation of these recalcitrant compounds are well studied and have been developed in pollution control. The degradative pathway of chlorinated benzenes is initiated by dioxygenases that produce chlorinated dihydrodiol intermediates, which are subsequently converted into the corresponding chlorocatechols by dihydrodiol dehydrogenases. The resulting chlorocatechols are oxidized by chlorocatechol dioxygenases, causing either ortho-cleavage to chloromuconic acid or meta-cleavage to 2-hydroxy-6-chlorocarbonil muconic acid. Chloromuconic acids are metabolized further to intermediates of the Krebs cycle.

Chlorocatechols are toxic to bacterial cells, therefore the regulation of the expression of these catabolic genes is very important for cell survival. These degradative pathways are usually regulated by LysR-type responsive transcriptional regulators (LTTRs), which are typically divergently transcribed from the structural genes. LTTRs are DNA-binding proteins that bind approximately 50–60 bp upstream of the genes they regulate. The presence of an inducer molecule, which is usually a catabolic intermediate of the pathway being regulated, alters the binding pattern and results in transcriptional activation. Examples of biodegradative pathways regulated by LTTRs in *Pseudomonas* include the chromosomally encoded catechol degradative *catBCA* operon and the *pheBA* operon (**Figure 2**), which allows the growth of the *P. putida* strain PaW85 on phenol. Both the *catBCA* and *pheBA* operons are regulated by CatR. Other examples include the 3-chlorocatechol degradative *clcABD* operon, regulated by ClcR, and the 1,2,4-trichlorobenzoate degradative *tcbCDEF* pathway in *Pseudomonas* sp. strain P51, regulated by TcbR.

In general, the genes that allow *Pseudomonas* to degrade aromatic compounds are likely recruited from preexisting catabolic pathways. The nature of the environments dictates to a large extent the mode of evolution of the new degradative pathways in microorganisms.

Polychlorinated biphenyl catabolism in recombinant Pseudomonas *strains*

Genes encoding polychlorinated biphenyls (PCBs)-degrading enzymes (*bph*) have been identified and isolated from several *Pseudomonas* species. PCBs, such as DTT, are toxic pollutants present in great abundance in the ecosystem, and bioremediation by soil bacteria has been extensively investigated in the last few decades. The catabolism of PCBs generally proceeds by the incorporation of both the atoms of oxygen (O_2) at the 2 and 3 positions of the least chlorinated ring, followed by 1,2-meta-cleavage of the molecule. PCBs are finally converted to a five-carbon aliphatic acid (2-hydroxypenta-2,4-dienoate), further degraded to chlorobenzoate, which accumulates in the growth medium. This dead-end product of the PCBs degradation can inhibit the bacterial growth and consequently slow down PCB biodegradation. To circumvent this limitation and to utilize *Pseudomonas* in bioremediation, recombinant strains are constructed by transferring the *bph* genes into *Pseudomonas* strains capable of utilizing several CBAs.

Metabolism of benzene, methylbenzene, and naphthalene by Pseudomonas

Pseudomonas have the potential to degrade aromatic hydrocarbons that range in size from a single ring (e.g., benzene, toluene, and xylene) to polycyclic aromatics (e.g., naphthalene). BTX aromatic compounds (benzene, toluene, and isomeric xylenes) usually occur together in gasoline and diesel oil. *Pseudomonas* degrade monoalkyl and dialkyl benzenes by different pathways, which include the oxidative attack on the aromatic ring and the formation of alkyl catechols, which are substrates for ring fission, or by the oxidation of alkyl substituents, which lead to the formation of aromatic carboxylic acids, further oxidized to dihydroxylated ring fission substrates. Subsequent conversion to the central metabolism intermediates proceeds through the meta-cleavage.

Naphthalene and its substituted derivatives are commonly found in crude oil and oil products. Naphthalene metabolism has been widely investigated in *Pseudomonas* as a model to understand the degradation of more complex polycyclic aromatic hydrocarbons (PAHs). PAHs are toxic and carcinogenic compounds so widely distributed in the environment to motivate the study of the microbial metabolism of these compounds to develop bioremediation technologies. *Pseudomonas* metabolizes naphthalene to salicylate, which is then converted to catechol, followed by ortho- or meta-cleavage to TCA cycle intermediates. In *P. putida* NAH7 plasmid, the genes encoding the enzymes involved in the naphthalene upper pathway and lower pathway are organized into two operons, *nah* and *sal*, respectively. Both the operons are turned on by NahR, a 36 kDa polypeptide and a salicylate-dependent transcription activator.

These catabolic pathways are mainly encoded on large plasmids such as the well-studied TOL plasmid pWWO, which is responsible for toluene and xylenes catabolism in *P. putida* and the naphthalene catabolic plasmid NAH7. These plasmids are generally conjugative, have low copy number, and undergo rearrangement and shuffling.

Degradation of alkanes and cycloalkanes in Pseudomonas

P. putida (*oleovorans*) can grow on *n*-alkanes by virtue of the alkane hydrolase system, which catalyzes the first step of alkane degradation, the oxidation of the methyl group to alcohol. The alkane hydrolase system consists of a membrane-bound alkane hydroxylase (*alkB*), a soluble electron transport system consisting of two rubredoxins, and a NADH-dependent rubredoxin reductase (encoded by *alkG*, *alkF*, and *alkT*, respectively). This system is investigated in great detail because of its industrial application in the production of fine chemicals, such as fatty acids, alcohols, and epoxides. The *alk* genes are mapped on its large catabolic OCT plasmid, which confers *Pseudomonas* the ability to degrade soluble short-chain alkanes, such as pentane, hexane, heptane, and octane, which are toxic for the environment and are produced by petroleum refineries.

Other interesting degradative activities in *Pseudomonas* are directed toward cycloalkanes, such as camphor or the highly toxic and persistent insecticide γ-hexachlorocyclohexane

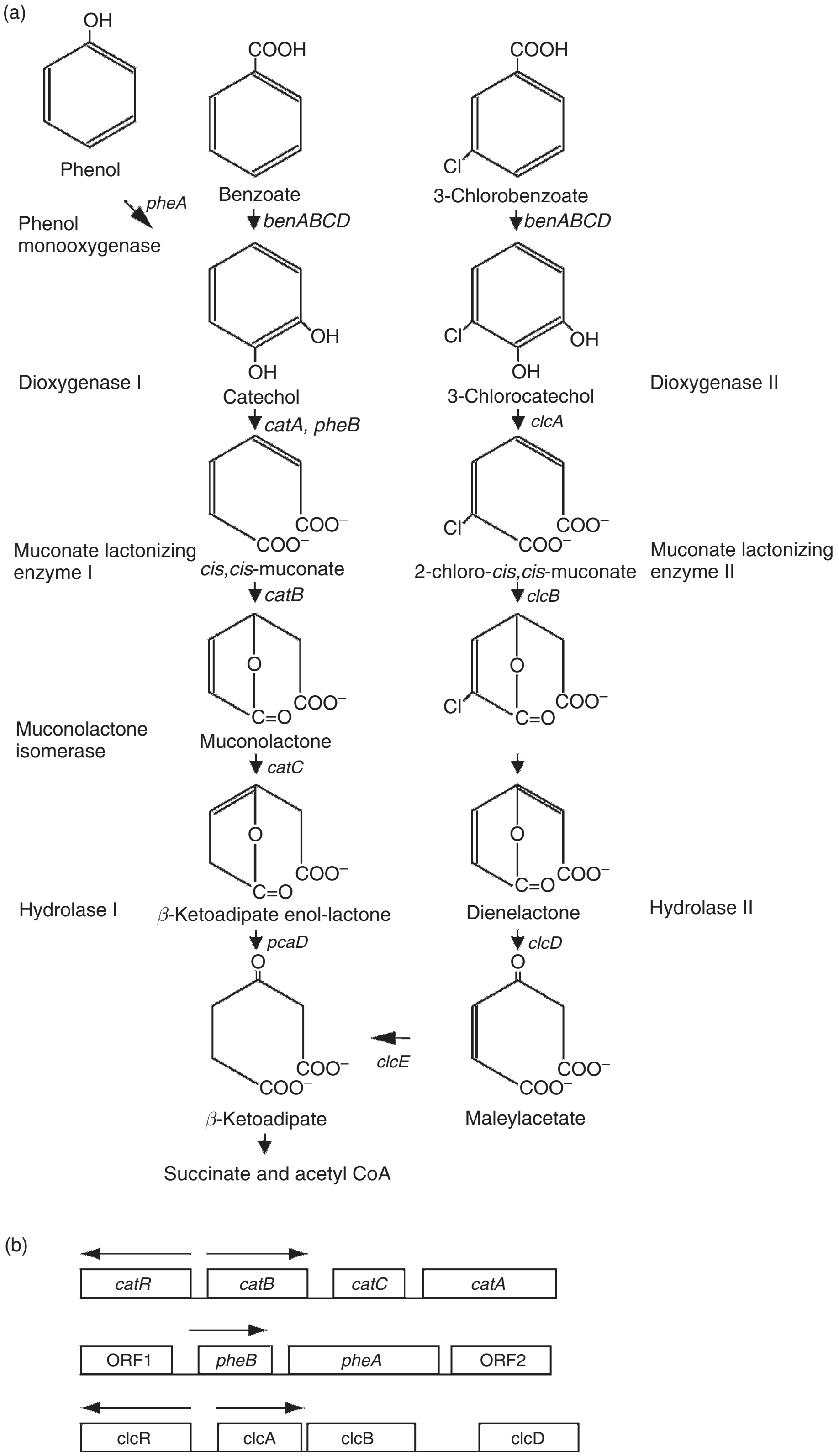
(a)
OH
Phenol
COOH
Benzoate
COOH
Cl
3-Chlorobenzoate
pheA
Phenol monooxygenase
benA BCD
benABCD
OH
OH
Catechol
Cl
OH
OH
3-Chlorocatechol
Dioxygenase I
Dioxygenase II
catA, pheB
clcA
COO−
COO−
cis,cis-muconate
Cl
COO−
COO−
2-chloro-cis,cis-muconate
Muconate lactonizing enzyme I
Muconate lactonizing enzyme II
catB
clcB
O
COO−
C=O
Muconolactone
Cl
O
COO−
C=O
Muconolactone isomerase
catC
O
COO−
C=O
β-Ketoadipate enol-lactone
O
COO−
C=O
Dienelactone
Hydrolase I
Hydrolase II
pcaD
clcD
O
COO−
COO−
β-Ketoadipate
clcE
O
COO−
COO−
Maleylacetate
Succinate and acetyl CoA
(b)
catR
catB
catC
catA
ORF1
pheB
pheA
ORF2
clcR
clcA
clcB
clcD

Figure 2 Enzymes and intermediates of the benzoate, phenol, and 3-chlorobenzoate degradation (a) and their genetic organization (b). *Pseudomonas putida* uses a modified β-ketoadipate pathway to degrade 3-chlorocatechol. The genes for the regulatory proteins CatR and ClcR are divergently transcribed from the *catBCA* and *clcABD* operons that they regulate. The *pheBA* operon is regulated by CatR.

(γHCH). *P. putida* PpG1, originally isolated by enrichment culture with D-camphor, carries the CAM plasmid, which encodes the enzymes necessary for D- or L-camphor degradation. Camphor is first converted to 5-exo-hydroxy camphor by a monoxygenase system consisting of three enzymes encoded by *camA*, *camB*, and *camC* genes. 5-exo-hydroxy camphor is then dehydrogenated to form 2,5-diketo camphane by F-dehydrogenase encoded by gene *camD*. These genes are organized in the *camDCAB* operon, which is negatively regulated by the product of the regulatory gene *camR*. *CamR* is located upstream of *CamD* and it is divergently transcribed. In the absence of camphor, CamR inhibits the expression of *camDCAB* and autorepresses the *camR* gene by binding to the operator between the regulator gene *camR* and the *camDCAB* operon. This inhibition is released in presence of the inducer camphor.

P. aeruginosa ITRC-5, isolated by selective enrichment on γHCH, can mineralize this insecticide. γHCH catabolic pathway has been comprehensively characterized for *Sphingomonas paucimobilis* UT26. This chlorinated insecticide is metabolized by the enzymes encoded by *linA*, *linB*, *linC*, *linD*, *linE*, and *linF* genes to β-ketoadipate, which is subsequently mineralized. Two or more copies of these genes are present in *P. aeruginosa* ITRC-5, which suggests that γHCH is degraded by *Pseudomonas* through a similar enzymatic pathway.

Pseudomonas *take part in the natural process of lignin mineralization*

Several members of the Pseudomonaceae have the ability to degrade lignin and the phenolic monomers, such as *trans*-ferulic, *p*-coumaric, and vanillic acid, which occur abundantly in the environment from the biodegradation of lignin accomplished predominantly by white-rot fungi. These products are utilized as a unique source of carbon and energy by *Pseudomonas*. The investigation of their degradative ability toward these lignin monomeric components is very important for bioremediation of pollutants, such as the chlorinated forms of vanillate, which are liberated in vast quantities into the environment by the wood pulp bleaching process.

Other environmental pollutants degraded by Pseudomonas

1. *Nylon*: Nylon is a polymer of 6-aminohexanoate (Ahx), widely used in the textile industry. During the polymerization, some molecules fail to polymerize and remain as oligomers and linear dimers (Ald) or undergo head-to-head condensation to form cyclic dimers (Acd). These nylon by-products are industrial waste products released in the environment. *Pseudomonas* sp. NK87 can grow on these compounds as sole source of carbon and nitrogen. This strain produces two hydrolases, Acd hydrolase and Ald hydrolases, encoded by *nylA* and *nylB* genes, respectively. These genes occur on catabolic plasmids present in *Pseudomonas* sp. NK87 and have evolved from other bacteria.
2. *Trichloroethane*: Trichloroethane (TCE) is widely used as degreasing agent, dry cleaning fluid, fumigant, and cleanser. Such wide use of TCE has caused it to become an environmental pollutant, especially in soils and groundwater. It is known to cause anemia and kidney and liver damage in humans. *P. putida* is capable of degrading TCE by producing a toluene dioxygenase. This enzyme converts TCE to glyoxylate or formate that are further metabolized.

Metal Resistance

Pseudomonas is resistant to a number of toxic metal ions, such as mercury, arsenic, cadmium, copper, chromium, and silver. Most of the resistant genes are plasmid-encoded and, occasionally, the regulatory genes are present on the chromosome.

Mercury resistance

Mercury is a toxic heavy metal. Resistant *Pseudomonas* species carry mercury-resistant (*mer*) determinants encoded on mobile genetic elements. The simplest *mer* determinants have been identified on transposon Tn501 in *P. aeruginosa*, where they are organized in the *merTPAD* operon. *MerR* is located upstream of the *merTPAD* and it is divergently expressed. MerR and MerD are involved in the regulation of the expression of the structural genes. MerR works as a *mer* operon inducer in the presence of Hg(II) and as a repressor in the absence of mercury salts. The *mer* operon encodes the transport proteins MerT and MerP; MerP is a periplasmic protein thought to scavenge Hg(II) in the periplasmic compartment to pass it to the inner membrane transporter MerT. From MerT, toxic Hg(II) is passed to mercuric reductase MerA. This NADPH-dependent cytoplasmic flavoenzyme detoxifies Hg(II) to volatile Hg^0.

Copper resistance

Copper (Cu) is a major micronutrient and it is a constituent of metalloenzymes and proteins involved in electron transport and redox reactions. However, it is extremely toxic at supraoptimal concentrations and it is also known to produce toxic free radicals. Two forms of Cu, Cu(I) and Cu(II), are normally found in bacteria. The well-studied copper resistance in *P. syringae* is due to a mechanism that involves copper binding and sequestration by plasmid-encoded proteins (*copABCD*). CopD is an inner membrane protein, which interacts with the outer membrane-associated protein CopB via the periplasmic proteins, CopC and CopA. The CopB, CopA, CopC, and

CopD proteins form a copper transport unit. CopS is a membrane-embedded copper-sensing protein and CopR is a DNA-binding protein, which activates the *cop* operon transcription. CopR and CopS form the two-component signal transduction system for sensing the levels of copper and regulating the *cop* operon.

In addition, copper tolerance in *P. fluorescens* and *P. aeruginosa* has been shown to be affected by a chromosome-encoded P1-type ATPase, which functions as an exporter of copper.

Cadmium resistance

Cadmium is very toxic to bacteria even when present in low concentrations, since it damages the cells by binding to essential respiratory enzymes, inducing oxidative stress or inhibiting DNA repair. *P. putida* produces a low-molecular-weight protein, which chelates cadmium, thereby reducing its toxicity. It also encodes a cadmium-transporting ATPase (CadA), an efflux system that confers resistance by reducing cadmium intracellular concentration. A cadmium and zinc efflux mechanism (*czr*), which is a cation–proton antiporter, rather than a cation-transport ATPase, was identified in the chromosome of *P. aeruginosa.*

Arsenic resistance

Arsenic is a top priority pollutant present in many ecosystems mainly in two oxidation states, arsenite [As(III)] and arsenate [As(V)]. Although some microorganisms can utilize arsenic, it generally is toxic to most bacteria. Arsenic resistance in *Pseudomonas* is due in part to the *ars* genes. Ars-mediated resistance involves As(V) reduction to As(III) via a cytoplasmic reductase (ArsC), and the As(III) is then extruded by a membrane-associated ArsB efflux pump.

Chromium resistance

Cr(III) and Cr(VI) are the most stable and abundant oxidative forms of chromium in nature and they are toxic to microorganisms. Cr(VI) is usually present as the oxyanion chromate, which crosses the biological membrane by means of the sulfate uptake pathway. Inside the cell, Cr(VI) is easily reduced to Cr(III), releasing free radicals that cause oxidative stress. *P. putida* minimize the toxic effect of chromate by means of a plasmid-encoded NADH-dependent reductase (ChrR). This flavin mononucleotide-binding protein reduces Cr(VI) to Cr(III) and, by an additional mechanism, reduces quinones, providing protection against free radicals generated by Cr(VI) reduction. *P. aeruginosa* ChrA protein is a chromate efflux pump, which represents an efficient mechanism of chromate resistance.

Silver resistance

Plasmids encoding silver resistance have been found in *Pseudomonas* strains isolated from silver mine or industrial sludge and in *P. aeruginosa* isolated from a patient in a burn unit after the topical use of silver compounds. However, silver resistance mechanism is not characterized yet.

Genetic and Molecular Tools Used to Study *Pseudomonas*

Plasmids

The utilization of nonenteric bacteria for basic and applied molecular research has resulted in the need for well-characterized vector systems for such microorganisms. The cloning vectors developed for this purpose are generally broad host range vectors and allow the use of different species, including *Escherichia coli*, as intermediary host.

General type cloning vector

There are many different broad-host-range vectors available for gene cloning in *Pseudomonas.* The majority of them are constructed based on existing replicons, such as RSF1010, RK2, or PRO1600, and inserting improved antibiotic resistance markers and additional cloning sites. Vectors such as pDSK509, pDSK519, and pRK415 are RSF1010-based vectors with the MCS from pUC19, kanamycin, or tetracycline resistance genes and the *lacZ* gene for easy screening of recombinant clones in *E. coli.* The pUC18/19 adaptation vectors, pUCP18 and pUCP19, were generated by introducing a pRO1600-derived stabilizing fragment into a pUC18/19 nonessential region to allow maintenance of these plasmids in *Pseudomonas* species. Many of these modified vectors are self-transmissible, whereas some have to be mobilized by triparental mating using a helper strain supporting *mob* functions *in trans.*

Special purpose cloning vectors

The expression of cloned genes in the host organism is often used to confirm the coding potential of a DNA fragment. However, large limitations are encountered when expressing cloned *Pseudomonas* genes in heterologous hosts, such as *E. coli.* These are primarily due to the differences in codon usage as well as due to variations in the structure of gene promoters. To overcome these problems, many laboratories have designed broad host-range expression vectors suitable for analysis of *Pseudomonas* genes. These vectors contain regulable promoters, such as the T7 promoter (P_{T7}) or the *E. coli lac* operon-based promoters P_{lac}, P_{tac}, and $P_{trc.}$ The first generation of controlled expression vectors pUCP18/19 offered regulated expression from P_{lac}, and two derivatives of pUCP19, pUCPKS and pUCPSK, were generated to allow expression from P_{T7}. A further development were the pBluescript-derived vectors, pBSP II SK(-) and pBSP II KS(-), which allow the controlled production of plasmid-encoded proteins from P_{T7} and P_{lac}. The pMMB family of expression vectors have been constructed using broad host-range vectors and the hybrid *trp-lac* (*tac*) promoter. A drawback of these controlled expression vectors in environmental applications is the high cost of the IPTG inducer. An alternative RSF1010-derived vector has been described that is based on P_m and P_u promoters of the *P. putida* TOL plasmid pWWO and the *xylS* gene, the product of which together with the coinducer benzoate positively regulates the *Pseudomonas* promoters.

For a quantitative analysis of the role and function of promoters in *Pseudomonas* species, gene fusion vectors containing the reporter genes *aphC* or *xylE* from RSF1010 or TOL plasmids, respectively, have been constructed. The first vector allows positive selection of promoters by selecting for streptomycin-resistant colonies, while the second allows for screening by catechol substrate. Other promoter probe vectors have been developed to identify *in vivo*-induced genes in *Pseudomonas* species with the *in vivo* expression technology (IVET), a method used to identify functions of ecological relevance or important for bacterial virulence and/or pathogenicity. *Ivi* genes can be identified by their ability to express a promoterless selection marker gene that is essential for survival *in vivo*. IVET system requires a strain that is a null mutant for the essential function encoded by the selection marker gene, an IVET plasmid carrying the promoterless selection marker gene, and a reporter gene. Several biosynthetic loci are essential for bacterial growth and have been exploited in the IVET systems. They include genes essential for the synthesis of purine and pyrimidine (*purA*, *purEK*, *pyrBC*) or for the synthesis of diaminopimelic acid (DAP), a component of the cell wall peptidoglycan (*asd*). ASD (aspartate β-semialdehyde dehydrogenase) is an essential enzyme in the biosynthesis of diaminopimelate, but it also plays a part in the biosynthesis of lysine, methionine, and threonine. As reporter genes, *lacZY* for β-galactosidase and *uidA* for β-glucuronidase have been incorporated into *Pseudomonas* IVET plasmids.

Transposable Elements

Transposable elements, including insertion sequences (ISs) and transposons, are common in various *Pseudomonas* species. The ISs have the capability of integrating into different sites of the genome in different bacteria since they contain the genetic determinants for transposition and short inverted repeat sequences (IRs) at both ends. During such an event, foreign genes are recruited by replicon fusion and insertional activation. Composite transposons can carry catabolic genes or antibiotic resistance markers flanked by two copies of similar ISs in direct or inverted orientation. However, transposons are also important tools for genetic analysis in *Pseudomonas*. They are used for generating gene disruption that is nonleaky and is linked to a selectable marker, or to deliver and stably incorporate genes in the chromosome for applications where plasmid cannot be readily maintained (e.g., environmental release). Recently, mini-Tn5 transposons are being used in several applications, including gene regulation studies and construction of strains for bio-remediation. These new delivery vehicles allow for single-copy chromosomal insertions and overcome the drawbacks of plasmid-based constructs, for example, high copy number or supercoiling, which interfere with promoter regulation studies, and antibiotic selection, which is not feasible with environmental release. However, these mini-transposons insert randomly in the chromosome and position effects cannot be easily controlled. To overcome this problem, the site-specific integration-proficient mini-CTX vectors were developed for *P. aeruginosa*. These vectors allow the insertion of gene cassettes at a defined location, the phage attachment 30 bp *attB* sequence, located at 2.94 Mb on the chromosome. Insertion at this naturally evolved phage integration site does not cause a mutant phenotype and does not compromise bacterial fitness. These mini-CTX vectors have been used in *P. aeruginosa* for gene expression from T7 and *lac* promoters or for promoter studies using *lac* and *lux*-based reporter genes. Ongoing genome sequencing projects are identifying possible *attB* sites in other *Pseudomonas* species to develop a wider use of this tool.

Proteomics and Microarrays

Pseudomonas genome sequencing projects have been completed for some species (*P. aeruginosa*, *P. fluorescens*, *P. stutzeri*, *P syringae*, *P. mendocina*, *P. entomophila*) and others are in progress. This genomic information is being complemented by global analysis of proteins expressed. This analysis is based on fractionation methods (e.g., cellular localization), protein identification and protein expression patterns comparison. The latter, mainly based on two-dimensional gel electrophoresis (2-DE) and multidimensional liquid chromatography, allows investigation of differential protein expression in response to one or more environmental or genetic variations. This comparative analysis has been used to detect differences among *P. aeruginosa* in chronic CF and in environmental isolates or among *P. aeruginosa* antibiotic-resistant and susceptible isolates. A proteomic study has been applied to investigate *P. putida* KT2440 biodegradation mechanisms by generating a proteome reference map for this strain grown in mineral salt medium and glucose as the only carbon source and comparing it to protein expression patterns after growth on different organic compounds.

The availability of sequenced genomes offers the possibility to develop DNA microarrays to investigate *Pseudomonas* environmental adaptation and pathogenesis. DNA microarrays are a tool to measure the simultaneous expression of thousands of genes in a single hybridization assay. Functional analysis of gene expression using microarray technology has been exploited to examine the global QS response and to characterize biofilm-regulated genes and antibiotic resistance of bacteria in the biofilm. Microarray studies have been extensively carried out in *P. aeruginosa* for which DNA chips have started to be commercially available. However, with increasing number of *Pseudomonas* strains being sequenced, microarrays can be tailored to study contaminant remediation in the environment or rizosphere colonization.

Biotechnology

Pseudomonas strains and their products have been used in large-scale biotechnological applications. *P. aeruginosa* PR3 is used in the conversion of surplus soybean oil to new value-added oxygenated products, including a compound with antifungal properties in controlling rice blast disease. Frostban is an ice minus *P. syringae*, used commercially to prevent ice nucleation in strawberry and potato fields. *P. putida* is used as a biocontrol agent for the *Fusarium* wilt pathogen to control black root rot disease of tobacco. Thermostable lipases from *P. fluorescens* are used in the food and leather industry. Polyesters produced by *P. oleovorans* are used in special plastics. *Pseudomonas* biosurfactants are used in emulsification, phase separation, emulsion stabilization, and viscosity reduction.

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Quorum-Sensing in Bacteria

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Glossary

AHLs Acylhomoserine lactones, quorum signal molecules produced by many Gram-negative bacteria.

autoinducer Molecule produced by a cell that causes self-regulation of pathways within the producing cell above a threshold concentration.

biofilm A community of bacteria associated with a surface.

bioluminescence The production of visible light by living organisms.

biomining The utilization of microorganisms to extract substances from rocks and minerals.

competence The ability of a cell to uptake and utilize DNA from outside sources.

conjugation The physical transfer of DNA from one organism to another.

lantibiotic Antibiotic peptides that contain the amino acid lanthionine.

mucinase Enzyme that degrades the glycoprotein material mucin.

orphan regulator Transcriptional regulators that are not part of a dedicated signal synthase/response system but can interact with the same signal.

synthase A class of enzymes that catalyze biosynthetic reactions of many compounds including quorum signals.

Abbreviations

AHL	Acylhomoserine Lactone
AIP	autoinducing peptide
CSP	competence-stimulating peptide
CT	cholera toxin
DPD	4,5-dihydroxy-2,3-pentanedione
EHEC	enterohemorrhagic *E. coli*
EPS	exopolysaccharide
GBAP	gelatinase biosynthesis-activating pheromone
HHQ	4-hydroxyl-2-heptyl-quinoline
LEE	locus of enterocyte effacement
PQS	Pseudomonas Quinolone Signal
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine
SRH	*S*-ribosylhomocysteine
TCP	toxin coregulated pilus

Defining Statement

Bacteria utilize numerous mechanisms to monitor and adapt to their external environment. One such mechanism involves the ability to 'count' their local population numbers. Once a specific number of cells, or quorum, is reached, bacteria are able to modify their group behavior through a mechanism known as quorum sensing.

Introduction

Bacterial growth and survival is dependent upon the ability of an organism to sense its environmental conditions and respond to external stimuli. Stimulus can come from a variety of sources including the nutrients available for growth, the presence of secondary metabolites, and the presence of other microorganisms. In the case of nutrients and secondary metabolites, sensor and regulatory proteins exist that affect a change in gene expression as these compounds change concentration. These changes in expression can cause bacteria to synthesize new proteins for catabolism in the case of diauxic growth, can lead to the production of extracellular enzymes to liberate nutrients from the environment in the case of starvation, or can cause changes in motility, chemotaxis, or metabolism that allow the bacteria to avoid or eliminate toxic concentrations of a secondary metabolite. In addition to these basic stimulus–response events, bacteria have evolved a mechanism to 'count' the local cell population. This process is known as quorum sensing and is mediated by the bacterium's ability to produce and recognize soluble factors known as quorum signals.

The ability of bacteria to utilize extracellular signals to modify their behavior in a cell density-dependent manner was first described in the early 1970s by Kenneth Nealson, Terry Platt, Woodland Hastings, and Anatol Eberhard. These researchers discovered that the bioluminescent bacterium *Vibrio fischeri* only produced light when bacterial cell numbers were high (**Figure 1**). They also demonstrated that culture supernatants from high cell density cultures were able to stimulate light production in low cell density cultures. This phenomenon was deemed autoinduction and provided the first clues that bacteria were able to utilize a soluble extracellular signal to monitor population density.

Subsequent work characterized the autoinducer of *V. fischeri* and the genes responsible for its synthesis and detection by the cell. As autoinducer-related genes were studied, it was discovered that many other Gram-negative bacterium contained the genes necessary for autoinducer synthesis and detection. It was demonstrated that autoinducer sensing was dependent upon a critical number of cells in a defined volume. This threshold density of cells was first referred to as a quorum by Clay Fuqua, Stephen Winans, and Peter Greenberg in 1994, and they proposed the term *quorum sensing* to describe this event.

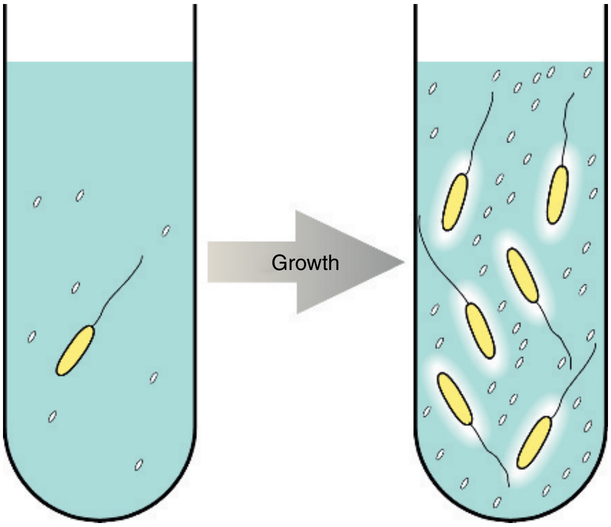

Figure 1 Cell density-dependent bioluminescence. As cells multiply, autoinducer concentrations increase. At a critical concentration, they induce synthesis of bioluminescence genes and subsequent light production.

Quorum sensing has rapidly expanded as a field of study, and the discovery of new quorum signaling bacteria as well as new types of quorum signals is increasing at an ever growing rate. Quorum signals in Gram-negative species such as *V. fischeri* were the first to be studied at the genetic and chemical levels; however, a significant amount of work has subsequently been performed with Gram-positive species. It is notable that quorum signal-dependent behavior was observed in Gram-positive bacteria long before the discovery of autoinducers or quorum sensing.

Quorum Signaling in Gram-Positive Bacteria

Although the molecular mechanism was unknown, the physiological effects of quorum signaling were known for a considerably long time in some Gram-positive species. Early work in bacterial genetics determined that some bacterial species were able to participate in horizontal gene transfer through natural competence. Several pioneering studies were done with organisms such as *Streptococcus pneumoniae* well before it was known that the process was quorum signal-dependent. Gram-positive species typically utilize small polypeptide signals as quorum signals. Some of these polypeptides undergo significant modifications to become a soluble extracellular signal (**Figure 2**).

Streptococcus pneumoniae ComC

S. pneumoniae is a common causative agent of pneumonia, otitis media, meningitis, and several other diseases. Genetic transformation studies were carried out as early as 1928 in this organism by Frederick Griffith. Subsequent work by Avery, McCarty, and MacLeod in 1944 demonstrated that genetic transformation in *S. pneumoniae* was due to uptake of extracellular DNA. Competency in *S. pneumoniae* is regulated by several factors but is primarily controlled by the competence stimulating peptide (CSP) quorum signal encoded by the gene *comC*.

Genetic competence in *S. pneumoniae* occurs during exponential phase and is initiated by CSP as cell density increases. The 17-amino acid CSP is produced by post-translational modification of the 41-amino acid precursor peptide ComC and is exported from the cell by the ComAB ABC transporter. When extracellular CSP reaches a threshold concentration due to increasing cell number, it is recognized by the sensor histidine kinase ComD. ComD autophosphorylates and subsequently transfers its phosphate group to the response regulator

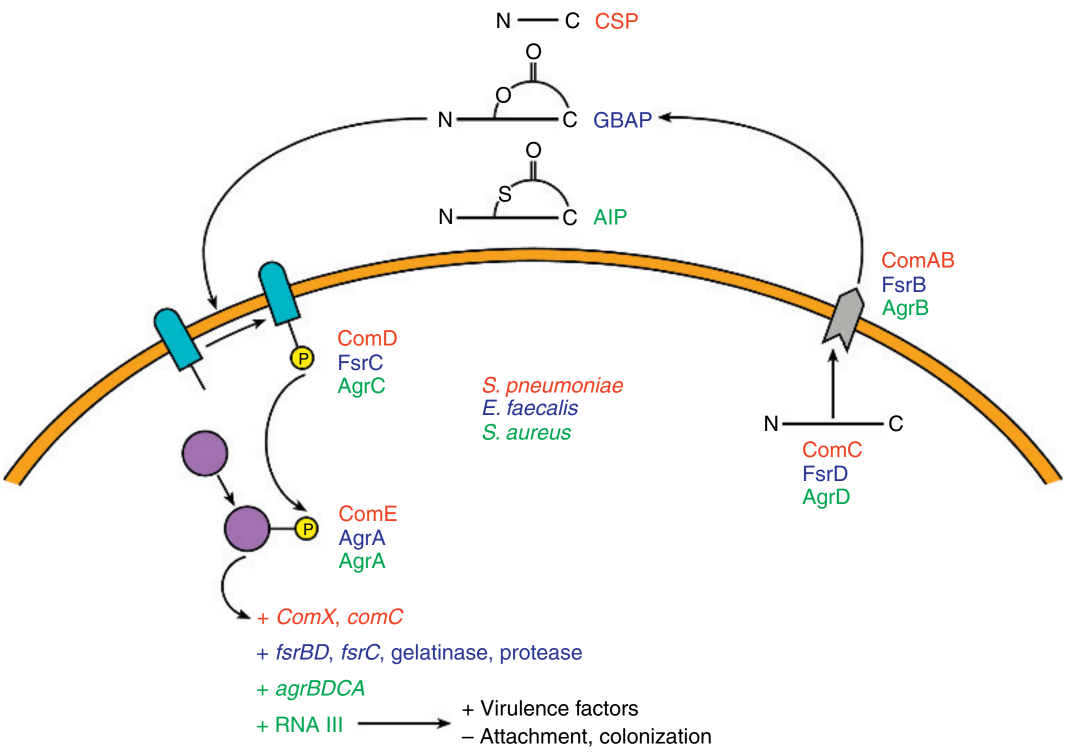

Figure 2 Quorum sensing peptides of *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Polypeptides ComC, FsrD, and AgrD are modified and exported out of the cell by the ComAB, FsrB, or AgrB transporters. Peptide autoinducers bind to the sensor kinases ComD, FsrC, or AgrC on the cell surface and initiate a phosphorelay to a cytoplasmic response regulator ComE or AgrA, which upregulates cell density-dependent gene expression.

ComE. Phosphorylated ComE activates transcription of the alternative sigma factor ComX, which goes on to induce expression of multiple genes involved in DNA uptake and recombination (**Figure 2**).

This peptide-induced, two-component signal transduction system is typical for Gram-positive quorum signaling circuits and was among the first to be characterized. Induction of competency at high cell density allows *S. pneumoniae* to sample and incorporate genetic material from its environmental counterparts. Surprisingly, *S. pneumoniae* competency permits the organism to take up DNA irrespective of its sequence or host origin. Such behavior gives *S. pneumoniae* the ability to adapt to selective pressures by taking up genes from neighboring cells that may have acquired genes or mutations that allow for enhanced fitness in a specific growth environment.

Enterococcus faecalis FsrB/D

E. faecalis is an opportunistic pathogen that causes endocardial, urinary tract, epidermal, and septic infections in clinical environments. *E. faecalis* is resistant to many common antibiotics and has recently acquired resistance to more modern antibiotic types making it a dangerous human pathogen. *E. faecalis* quorum sensing uses a cyclic lactone-modified peptide signal known as the gelatinase biosynthesis-activating pheromone (GBAP). GBAP is an 11-residue, circular polypeptide closed by a lactone moiety not seen in other Gram-positive-produced cyclic peptides. GBAP is not produced by all *E. faecalis* strains, but its production has been observed in all strains that produce the virulence factor gelatinase.

Originally, the *E. faecalis* GBAP signal was thought to be derived from the C-terminal region of the 212-amino acid FsrB protein. Recent studies have shown that a previously unknown gene, *fsrD*, exists in frame with *fsrB*, and GBAP is derived from FsrD by proteolytic activity of the FsrB protein. The FsrB protein was originally thought to autocatalyze by simultaneously forming and modifying GBAP while exporting it out of the cell. This new data show that the N-terminal region of FsrB is necessary for modification of the previously unknown FsrD peptide to produce the mature GBAP signal.

GBAP is produced maximally at the end of exponential phase in *E. faecalis*. When GBAP concentration reaches a density-dependent threshold, it induces autophosphorylation of the sensor kinase FsrC followed by phosphotransfer to the AgrA protein. Phosphorylated AgrA is a response regulator that promotes expression of *fsrBD*, *fsrC*, and the virulence factors gelatinase and serine protease (**Figure 2**). Recent transcriptome analyses of *fsrBD* mutants have shown indirect regulation of genes involved in biofilm formation, surface protein synthesis, and carbon source uptake, as well as direct regulation of an uncharacterized open reading frame. Interestingly, GBAP production is critical for successful colonization in an animal model. It is hypothesized that density-dependent regulation of gelatinase and serine protease by GBAP at the onset of stationary phase allows *E. faecalis* to liberate nutrient sources in the host as they become limiting.

Staphylococcus aureus AgrD

S. aureus is a human pathogen that causes toxic shock syndrome, food poisoning, and epidermal and endocardial infections. The prevalence of antibiotic-resistant *S. aureus* strains has placed it among the most commonly encountered nosocomial infections. *S. aureus* uses the accessory gene regulation (*agr*) system to regulate numerous genes involved in virulence and colonization in a density-dependent fashion via an autoinducing peptide (AIP) signal.

AIP is a thiolactone containing octapeptide ring derived from the 46-amino acid protein AgrD. To form AIP, AgrD is proteolyzed, modified by addition of a thiolactone moiety and exported from the cell by AgrB. Extracellular AIP is detected by the AgrC sensor histidine kinase, which autophosphorylates when bound to AIP. Phospho-AgrC transfers its phosphate group to the response regulator AgrA. The AgrC-AgrA two-component sensor kinase system upregulates the *agrBDCA* operon, establishing a positive feedback loop at sufficient extracellular AIP concentrations. AgrA also induces transcription of the regulatory RNA, RNAIII. RNAIII is an untranslated RNA that upregulates many *S. aureus* virulence factors including toxins and extracellular proteases while downregulating low-density genes involved in attachment and colonization (**Figure 2**). Thus, AIP signaling provides *S. aureus* with the ability for transition from colonization and survival at low cell density to pathogenesis and nutrient acquisition at high cell density. This behavior has been hypothesized to allow *S. aureus* to establish a colonization site in the host before virulence factor activity stimulates the host-immune response.

The *S. aureus* quorum sensing and response pathway is very similar to the one found in *E. faecalis*; however, there are notable differences including an increased number of AIP-regulated genes as well as strain specificity of the AIP signal. *S. aureus* AIP has been shown to modulate expression of a larger number of genes, a feat accomplished by induction of RNAIII, and other transcriptional regulators. AIP signaling has also been shown to be strain-specific. There are four known *agrD* specificity groups in *S. aureus* strains. AIP from a single specificity group has been shown to inhibit the AgrC sensor kinase of other groups. Thus, *S. aureus* has acquired a way to compete against other strains of its own species in an infection site when an individual strain reaches a threshold cell density. Therefore, the first strain in a single specificity group to achieve autoinduction of the *agr* pathway will induce its own virulence factor production and block quorum signaling activity in other strains. Quorum sensing systems similar to AIP are present in other *Staphylococcus* species, and these systems exhibit group strain competition as well.

Bacillus subtilis ComX and Phr Peptides

B. subtilis is a soil bacterium that can undergo spore formation or display genetic competence upon entry into stationary phase. Roughly 10% of *B. subtilis* cells entering stationary phase become competent. Competency in this situation is thought to aid *B. subtilis* in DNA repair and contribute to the inheritance of genetic material from *B. subtilis* strains that have successfully grown to high cell density. The decision to become competent or to sporulate is influenced by many factors in a complex regulatory pathway through which quorum signals play a significant part.

The ComX peptide is a 10-amino acid linear peptide derived from a 55-amino acid precursor product of the *comX* gene. The original ComX polypeptide is cleaved, modified, and exported out of the cell by the ComQ protein. Extracellular ComX is sensed by the ComP sensor kinase. Above threshold concentrations of extracellular ComX, ComP phosphorylates the response regulator ComA, which induces the *comS* gene. ComS blocks proteolysis of the transcriptional activator ComK, which upregulates the production of many genes that stimulate competency in the host cell (**Figure 3**).

A second quorum signaling pathway utilizes multiple proteinaceous pheromone (Phr) signals in a complex regulatory circuit (**Figure 3**). Phr precursors are proteolyzed and exported out of the cell as linear pentapeptides. Extracellular Phr is taken up into the cell by an ABC oligopeptide transporter. Multiple Phr genes exist and serve to antagonize the activity of several Rap phosphatase enzymes, which regulate many steps along the sporulation and competence pathways. For example, extracellular PhrA enters *B. subtilis* and binds directly to the RapA regulatory phosphatase either preventing it from binding or disassociating it from bound Spo0F-phosphate. RapA bound to the intermediate response regulator Spo0F-phosphate prevents a phosphorelay cascade to the global response regulator Spo0A, thereby preventing sporulation. Thus, increased PhrA levels are one of many factors that allow sporulation to begin by interrupting Spo0F inhibition of Spo0A. In addition to PhrA, there are several other Phr-type signals that are recognized by *B. subtilis* at the end of exponential phase and help direct the organism to specialize in sporulation or competence.

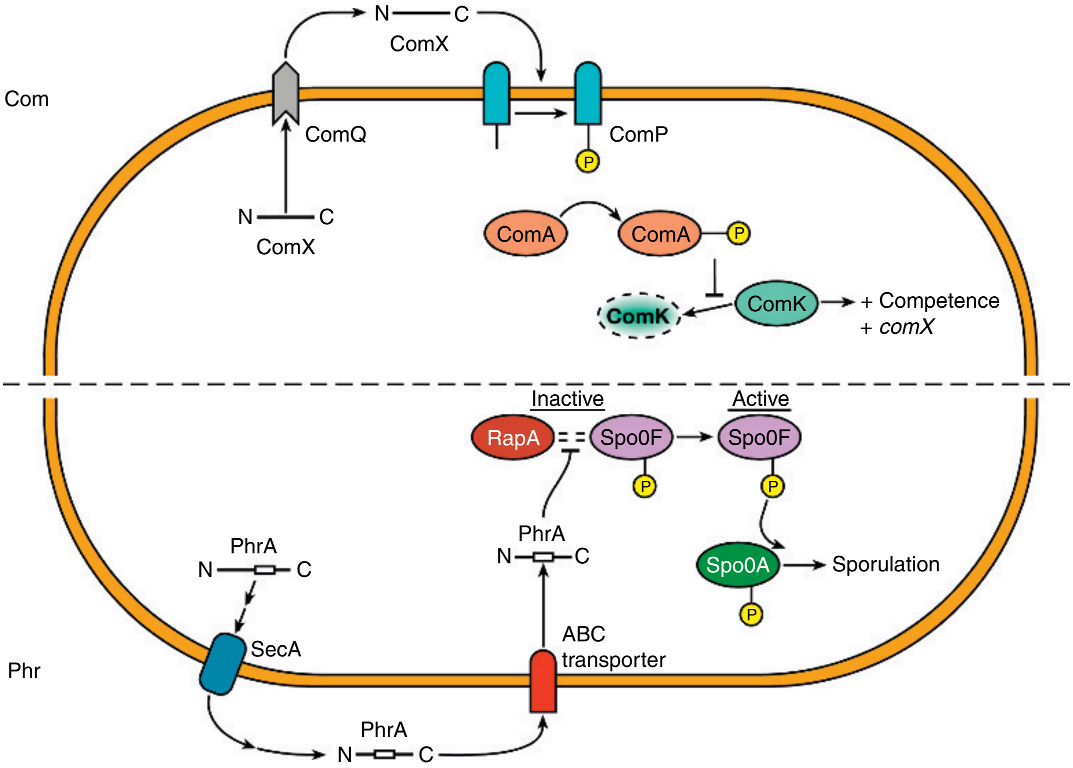

Figure 3 Quorum sensing pathways in *Bacillus subtilis*. In the Com system (upper panel), the ComX protein is cleaved, modified, and exported out of the cell by the ComQ transporter. Mature ComX interacts with the ComP sensor kinase that phosphorylates ComA. ComA-phosphate blocks the turnover of the transcriptional regulator ComK, which induces *comX* and competence gene expression. The Phr system (lower panel) utilizes the PhrA protein, which is cleaved and exported out of the cell using the Sec transport system. Mature PhrA returns to the cytoplasm via an oligopeptide ABC transporter and interferes with RapA binding to Spo0F-phosphate. Spo0F-phosphate can then phosphorylate the response regulator Spo0A, which induces sporulation genes.

Lactococcus lactis Nisin

L. lactis is a fermentative bacterium commonly used in the dairy industry to produce buttermilk and cheese. *L. lactis* is classified as a lactic acid bacterium as it produces large amounts of lactate upon fermentation of sugars found in dairy products. *L. lactis* produces the antimicrobial peptide nisin, which was discovered in 1928 due to its ability to inhibit growth of other lactic acid bacteria. In 1988, nisin was approved by the FDA for use as a preservative agent in the food industry.

Nisin is a Class I bacteriocin or lantibiotic characterized by its small size and the inclusion of the amino acids lanthionine and β-methyllanthionine as well as other dehydrated amino acids. Nisin is bactericidal and forms pores in bacterial membranes. This activity is especially effective against other Gram-positive bacteria where cell membrane perforations immediately cause a loss of membrane integrity.

Despite its role as an antimicrobial agent, nisin also serves as a density-dependent signal for *L. lactis*. Nisin is a highly modified 34-amino acid product of the 57-amino acid polypeptide NisA. NisA is modified and exported out of the cell by the NisBCT membrane-associated complex. Upon leaving the cell, modified NisA is cleaved to form nisin by the extracellular NisP protease. Damage of the parent cell membrane by nisin is prevented by the production of several proteins that block nisin activity at the cell surface. Extracellular nisin concentration is sensed by the NisK sensor kinase, which phosphorylates and activates the NisR response regulator. Nisin production is maximal during early stationary phase growth. NisR promotes expression of the *nisA* gene as well as genes that encode for proteins that block nisin activity at the cell surface, allowing for a positive feedback loop to upregulate nisin production as *L. lactis* enters stationary phase. Interestingly, the production of nisin demonstrates the ability of a compound to act as both a quorum signal and an antimicrobial agent. This type of dual function by an extracellular compound provides *L. lactis* with an efficient and powerful quorum signaling system that might provide a notable competitive advantage in polymicrobial environments.

Acylhomoserine Lactone (AHL) Quorum Signals in Gram-Negative Bacteria

The distribution of Gram-negative and Gram-positive bacterial species throughout the world is highly similar. Both types of organisms inhabit nearly every known niche on the planet and are involved in biogeochemical mineral cycles, biochemical degradation, disease, and the production of a staggering number of extracellular small molecules. While Gram-positive species have largely evolved to utilize polypeptide quorum signals that depend on cell-surface receptors, Gram-negative bacteria often utilize soluble signals known as AHLs. Many AHLs are able to pass through the lipid bilayer and are therefore able to interact with cytoplasmic regulatory proteins; thus, AHLs do not rely on the phosphorelay cascades that Gram-positive quorum sensing pathways commonly use. Both types of signals are effective due to their stability and solubility in environments colonized by the organisms that synthesize them.

V. fischeri LuxI/R

As mentioned previously, quorum signaling was discovered in the luminescent marine bacteria *V. fischeri* and *Vibrio harveyi*. In the early 1970s, researchers observed that supernatants from stationary phase cultures could be added to cells at low density and trigger light production. This suggested the presence of an autoinducing compound made in later phases of *V. fischeri* and *V. harveyi* growth. Further study determined that the factor in spent medium was relatively species-specific and dependent on cell density rather than the nutritional status of the cells. In 1981, the *V. fischeri* autoinducer was purified and determined to be the AHL 3-oxo-hexanoyl-HSL ($3OC_6$-HSL). A variety of AHL compounds have been characterized from other organisms and are collectively referred to as autoinducer-1 (AI-1).

V. fischeri can be found free-swimming and can also participate in a symbiotic relationship with the Hawaiian bobtail squid *Euprymna scolopes*. *V. fischeri* colonizes a cavity on the squid host known as the light organ. *V. fischeri* receives nutrients from the host in the light organ and emits light as its population density and AI-1 concentrations increase. Luminescence from the light organ of *E. scolopes* is thought to help the squid evade predation by masking the organisms' shadow in shallow water. *V. fischeri* has also been observed to enter symbiotic relationships with other marine organisms such as the fish *Monocentris japonicus* where a *V. fischeri*-dependent light organ is used to attract a potential mate.

The *V. fischeri* LuxI protein synthesizes 3-oxo-C6-HSL from *S*-adenosylmethionine (SAM) and acylated acyl carrier protein. Once synthesized, $3OC_6$-HSL freely diffuses across the membranes and out of the cell. At a particular density, a critical concentration of AI-1 is reached that stimulates AI-1 to interact with and activate the response regulator LuxR. Activated LuxR promotes transcription of the *luxR* gene as well as the *luxICDABEG* operon, which serves to produce light and generate more AI-1 (**Figure 4**). Thus, a positive feedback loop develops at sufficient cell densities, which allows for a substantial increase in light production. AI-1 exists in many Gram-negative species and is thought to function as an intraspecies-specific signal.

Recently, a second AHL quorum signal has been identified in *V. fischeri* and has been shown to function as part of a regulatory network in combination with $3OC_6$-HSL and a third signal, the furanosyl borate diester signal AI-2 (which will be discussed later). This AHL signal is *N*-octanoyl HSL (C_8-HSL) and is produced by AinS (**Figure 4**). C_8-HSL regulates luminescence at low culture densities by relieving negative regulation of the LuxR protein due to inactivation of the transcriptional regulator LuxO. Inactivation of LuxO allows upregulation of *litR*, whose gene product serves to activate *luxR* transcription. C8-HSL also interacts with LuxR directly, allowing for initial activation of the *luxICDABEG* operon.

Pseudomonas aeruginosa Las/Rhl AI-1 System

P. aeruginosa is a Gram-negative bacterium commonly isolated from soil environments, which is frequently used as a model organism to study quorum sensing. *P. aeruginosa* is an opportunistic pathogen that can cause an array of infections in immunocompromised individuals, most notably those inflicted with the heritable disease cystic fibrosis. Many of the genes necessary for infection, nutrient acquisition, virulence factor production, and biofilm growth are regulated by the concentrations of two AHL signals produced by LasI and RhlI. Due to its effects on virulence factor production, interference with quorum signaling in *P. aeruginosa* has been proposed as a therapeutic strategy. This novel approach to antimicrobial therapy in a notoriously antibiotic-resistant organism makes further study of quorum sensing in *P. aeruginosa* increasingly important.

The LasI protein synthesizes the signal *N*-(3-oxododecanoyl)-L-HSL ($3OC_{12}$-HSL). $3OC_{12}$-HSL interacts with the LasR response regulator, which upregulates the expression of multiple genes involved in virulence such as the *lasB* protease as well as the *lasI* gene itself, establishing $3OC_{12}$-HSL as an autoinducer. While $3OC_{12}$-HSL is diffusible, it can also partition into the cell membrane and has been shown to be exported by efflux pumps in *P. aeruginosa.*

The LasR-$3OC_{12}$-HSL complex serves to upregulate a second AHL-dependent system by inducing expression

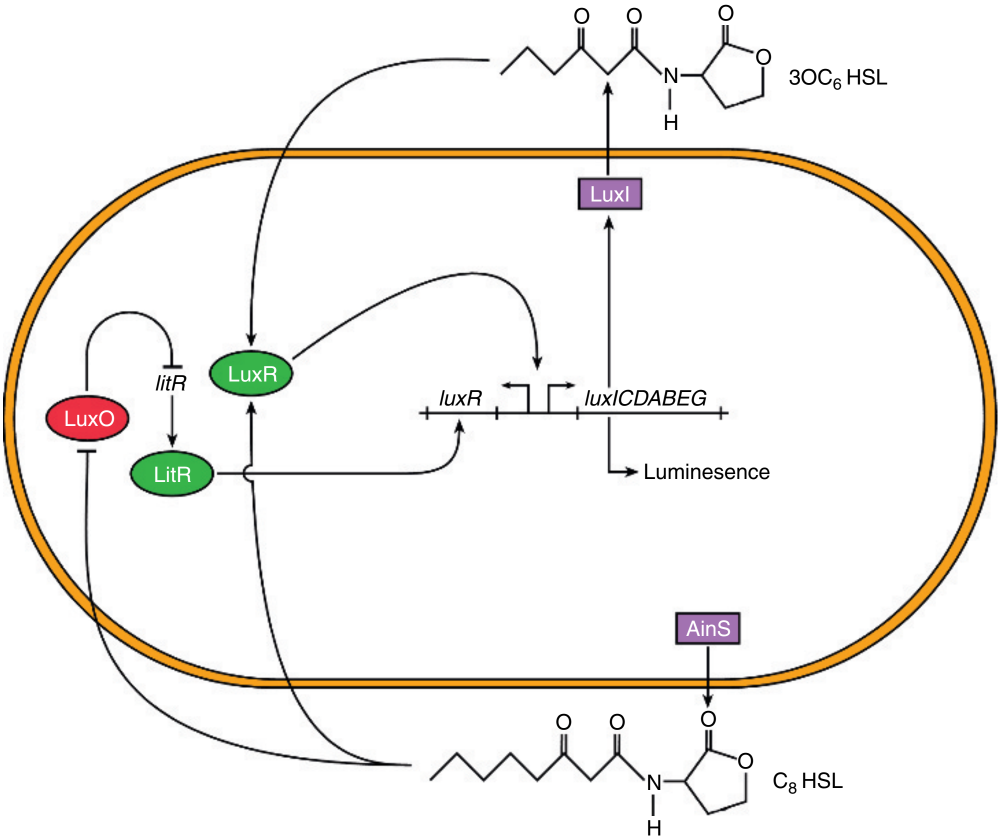

Figure 4 Quorum sensing in *Vibrio fischeri*. The LuxI-produced 3OC$_6$-HSL diffuses out of the cell until it reaches sufficient concentration to induce the LuxR response regulator. 3OC$_6$-HSL-LuxR induces expression of the *luxR* and the *luxICDABEG* genes, which increase synthesis of 3OC$_6$-HSL as well as proteins involved in bioluminescence. At low cell density, C$_8$-HSL, produced by the AinS gene, provides initial activation of LuxR by direct activation as well as by inhibiting LuxO, preventing the *litR* gene product from inducing the *luxR* gene, thereby increasing levels of *luxR* transcript in the cell.

of *rhlR* (**Figure 5**). RhlI synthesizes *N*-butyryl-L-HSL (C$_4$-HSL), which interacts with the response regulator RhlR to regulate numerous genes including those involved in secondary metabolite production, as well as the *rhlI* gene that establishes a second AHL autoinduction loop. C$_4$-HSL-dependent regulation overlaps with 3OC$_{12}$-HSL-mediated regulation in the control of several genes such as the extracellular protease encoded by the *lasB* gene. It is notable that 3OC$_{12}$-HSL inhibits the interaction of C$_4$-HSL with its response regulator RhlR while inducing expression of the *rhlR* gene. This interaction serves to maintain levels of C$_4$-HSL low until RhlR concentration increases to levels sufficient to interact with the low levels of C$_4$-HSL produced in late exponential phase. Thus, the activation of the C$_4$-HSL–RhlI feedback loop becomes functional only after LasR-3OC$_{12}$-HSL-dependent regulation has occurred.

Both quorum signals in *P. aeruginosa* ultimately serve to regulate the expression of over 300 genes involved in diverse pathways. Such regulation is not achieved by the LuxR-like LasR and RhlR response regulators alone. For example, LasR induces expression of the *vqsR* gene, whose product upregulates genes involved in virulence factor production and quorum signaling. Apart from downstream regulators induced by LasR or RhlR, ‘orphan’ LuxR-type regulators that do not have a cognate LuxI-type produced AHL signal have been discovered. QscR is a LuxR-type response regulator that binds the promoters of several genes in the presence of the LasI-generated signal 3OC$_{12}$-HSL. Apart from these interactions, QscR has been shown, at least indirectly, to repress *lasI* expression (and was in fact named for this activity, quorum signaling control repressor). The manner in which QscR represses *lasI* expression is unclear but has been hypothesized to involve heteromultimerization of QscR and LasR. Another hypothesis is that QscR binding to 3OC$_{12}$-HSL prevents this AHL from coming in contact with its cognate regulator LasR. Interestingly, QscR has also been shown to be more promiscuous in AHL binding than either LasR or RhlR, suggesting a role in interspecies AHL sensing. Ultimately the HSL-dependent quorum signaling network combined with orphan response regulators contribute a great deal to global gene regulation in *P. aeruginosa*.

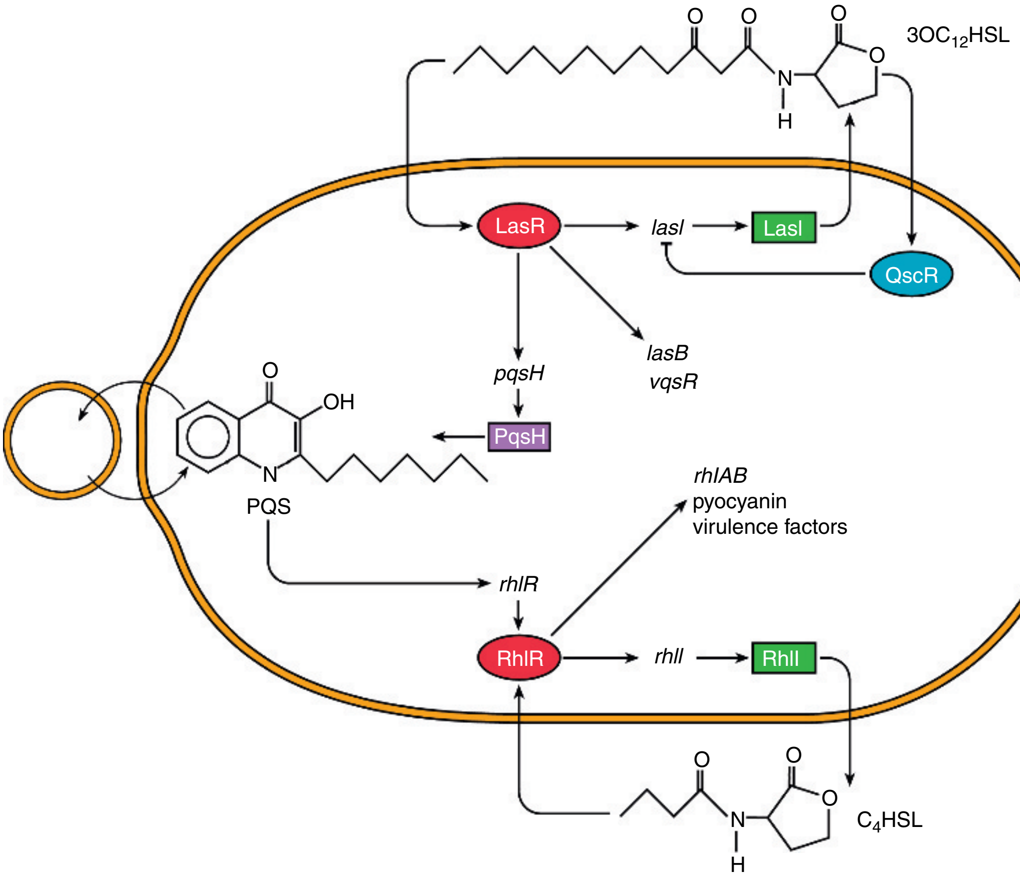

Figure 5 Quorum sensing in *Pseudomonas aeruginosa*. 3OC$_{12}$-HSL produced by the LasI synthase interacts with the LasR transcriptional regulator, which upregulates *lasI*, *lasB*, *vqsR*, and *pqsH*. 3OC$_{12}$-HSL also interacts with the orphan transcriptional regulator QscR to repress *lasI* expression. PqsH produces the quinolone signal PQS that is transported between cells via outer membrane vesicles. PQS induces expression of the *rhlR* gene whose product induces expression of the *rhlI* synthase gene as well as several other virulence factors when induced by the RhlI-produced C$_4$-HSL signal.

Agrobacterium tumefaciens Tra System

A. tumefaciens is a Gram-negative soil bacterium, which is the causative agent of crown gall disease in plants. Some *A. tumefaciens* strains carry a Ti (tumor-inducing) plasmid that contains the LuxI/R-type quorum signaling genes *traI* and *traR*. The Ti plasmid also carries genes that encode for specific plant hormones. After conjugation of the Ti plasmid from *A. tumefaciens* to the host nucleus, plants synthesize these hormones that then stimulate cell proliferation and tumor formation.

Free-swimming *A. tumefaciens* move throughout the soil by flagellar motility and search for susceptible hosts by chemotaxis toward metabolic intermediates released from wounded plants. Upon contacting the host, *A. tumefaciens* produces cellulose fibrils as well as attachment proteins to anchor it to host tissue. After attachment, *A. tumefaciens vir* (virulence) genes are induced by host-derived compounds that in turn activate synthesis of a secretion apparatus that directs translocation of the Ti plasmid from *A. tumefaciens* into the host. Ti plasmid-encoded hormones then cause cell proliferation and tumor formation. The Ti plasmid also contains genes whose products direct synthesis of opines. Opines are specialized amino acids that *A. tumefaciens* uses as a growth substrate. There are at least two known classes of opine-encoding genes, octapine and nopaline, and *A. tumefaciens* strains or pathovars can be classified by which genes are present on the Ti plasmid. These plasmid-borne genes not only direct host synthesis of opines, but contain opine catabolism genes, allowing *A. tumefaciens* to use opines as a source of carbon and energy.

A. tumefaciens uses the TraI/R quorum sensing system to regulate conjugation of the Ti plasmid between *A. tumefaciens* strains. The *traI/R* quorum signaling genes are similar to the *luxI/R* genes from *V. fischeri*, but there are key differences in regulation. In octapine-utilizing pathovars, the *A. tumefaciens* transcriptional activator OccR is induced by the presence of the opine octapine. OccR positively regulates expression of *traI*, whose product synthesizes the *N*-(3-oxo-octanoyl)-L-HSL (3OC$_8$-HSL) quorum signal. In nopaline-utilizing

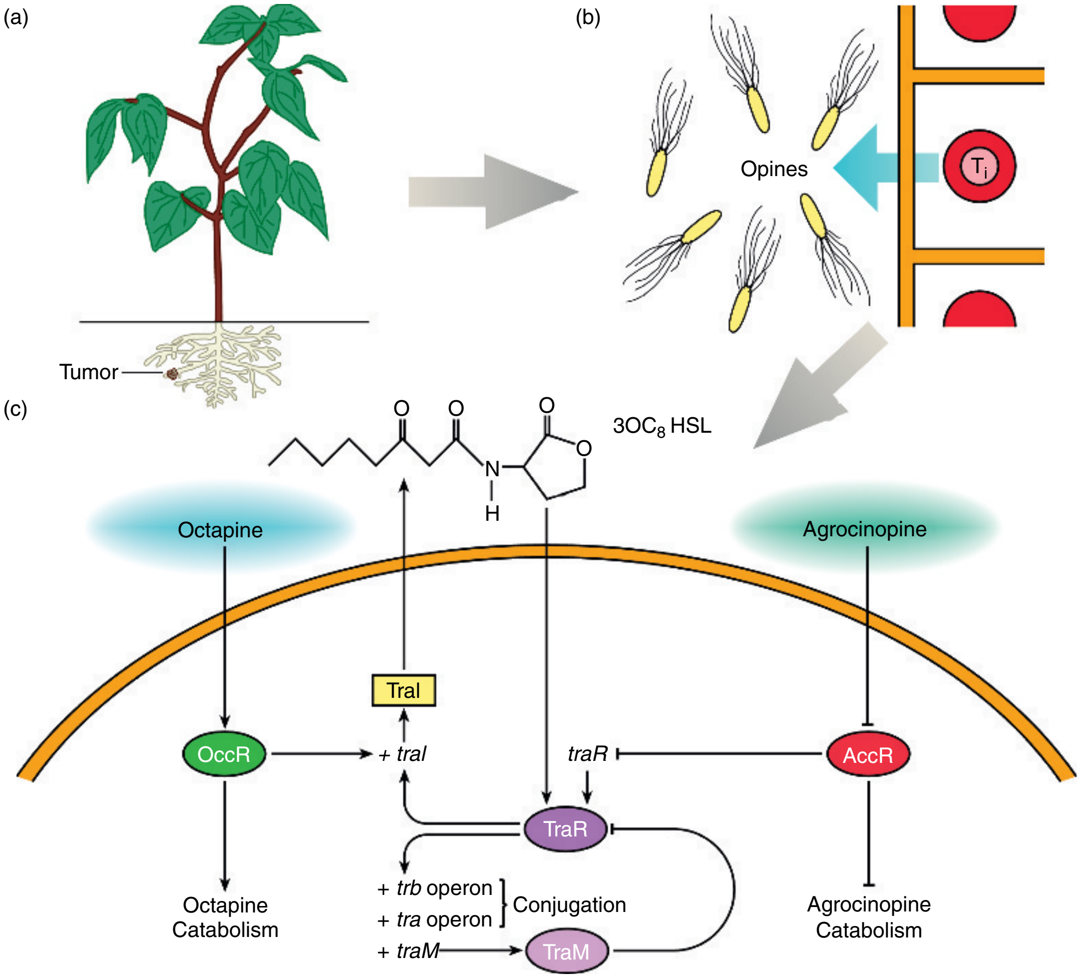

Figure 6 Quorum sensing in *Agrobacterium tumefaciens*. (a) A plant infected with *A. tumefaciens* displaying a characteristic tumor. (b) Inside the tumor, proliferating host cells that carry the Ti plasmid produce an opine nutrient for *A. tumefaciens*. (c) Octapine-type opines interact with the OccR transcriptional regulator causing it to upregulate octapine catabolism genes as well as induce *traI* expression. TraI synthesizes the $3OC_8$-HSL signal that interacts with the TraR protein, causing it to further induce *traI* expression and the *trb* and *tra* operons utilized in conjugation. $3OC_8$-HSL-TraR also induces the *traM* gene, whose product inhibits TraR activity.

pathovars, the opine agrocinopine inactivates the *traR* repressor AccR. This interaction also increases expression of *traI* in the cell (**Figure 6**).

Increased *traI* expression in the cell ultimately leads to an increase in $3OC_8$-HSL. As $3OC_8$-HSL reaches a critical threshold, it induces *traR* expression. When TraR is bound to $3OC_8$-HSL, it induces *traI*, establishing an autofeedback loop. Activated TraR also induces expression of the *trb* operon, which produces the mating pore between two *A. tumefaciens* cells; the *tra* operon, which helps mobilize the Ti plasmid; and the *traM* gene. TraM inactivates TraR, thus providing a means for *A. tumefaciens* to downregulate the quorum signal-dependent synthesis of the conjugation apparatus after it has begun. The *A. tumefaciens* TraI/R system, while similar to the LuxI/R system of *V. fischeri*, has an additional level of regulation dependent on host opine production ensuring that quorum signal-controlled induction of conjugation does not occur prematurely outside of a compatible host. This system serves as an elegant example of interspecies-dependent cues having regulatory input in bacterial signaling pathways.

Pantoea stewartii EsaI/EsaR System

The Gram-negative bacterium *P. stewartii* is the causative agent of Stewart's bacterial wilt and leaf blight in sweet corn and maize. *P. stewartii* is spread by infected populations of the corn beetle, *Chaetocnema pulicaria* that introduces the bacterium into the xylem and intercellular leaf spaces of the plant during feeding. Infection by *P. stewartii* results in wilting due to colonization of the xylem and formation of water-soaked lesions due to bacterial growth within young leaves. Buildup of large amounts of exopolysaccharide (EPS) results in vascular occlusion of plant tissue. EPS secreted by *P. stewartii* is the principal virulence factor and is part of a multistep invasion process.

P. stewartii secretes EPS in a cell density-dependent fashion. Biosynthesis of EPS is encoded by the *cps* gene cluster, while regulation of EPS synthesis is mediated partly by the EsaI/R quorum signaling system. The EsaI/R system also controls the Hrp (hypersensitivity and pathogenicity) regulon. *P. stewartii* requires the Hrp type III secretion system for infection of the intercellular leaf spaces and formation of the characteristic water-soaked lesions.

The *cps* genes are regulated by the Rcs (*r*egulator of *c*apsule *s*ynthesis) two-component signal transduction system that detects environmental signals. The Rcs system is composed of the RcsB cytoplasmic response regulator and the RcsC transmembrane sensory protein. It is thought that for complete induction of the *cps* genes, another protein (RcsA) may be required. Also, in the absence of AHLs, EsaR negatively regulates the *cps* genes.

Interestingly, the AHL synthase gene *esaI* is constitutively expressed and is not autoregulated by *esaR* like many other *luxI*/*R*-type genes, while *esaR* is autorepressed by the EsaR protein. EsaI synthesizes 3-oxo-C_6HSL and small amounts of 3-oxo-C_8HSL. Mutants in *esaI* do not produce AHLs or EPS and are avirulent. In contrast, *esaR* and *esaI*/*R* double mutants demonstrate a constitutive hypermucoidy phenotype and are less virulent than the wild type. The hypermucoidy mutants of *P. stewartii* also appear impaired in attachment compared to wild-type *P. stewartii.* This indicates the importance of quorum control of pathogenicity factors in *P. stewartii* as production of EPS at an incorrect location and phase of infection renders the cells avirulent and unable to colonize the host.

Acidithiobacillus ferrooxidans AfeI/R

A. ferrooxidans is a Gram-negative acidophilic chemolithotrophic bacterium that is commonly found in multispecies biofilms on mineral surfaces such as pyrite and elemental sulfur in rock and soil. *A. ferrooxidans* catalyzes the oxidation of iron and sulfur yielding sulfuric acid. It is a causative agent of acid mine drainage, which can lead to groundwater contamination. The ability of *A. ferrooxidans* to dissolve mineral structures can also be used in ‘biomining’ where acid solubilization of rocks and soil can release minerals such as copper and gold that are used in industrial applications. This process, also known as bioleaching, is a slower yet more energy efficient and environmentally containable process as opposed to traditional smelting of ores to release rare metals.

To date, AHL quorum signaling in *A. ferrooxidans* is relatively poorly understood. Recent studies have determined that *A. ferrooxidans* produces nine different medium to long-chain (C8–C16) AHL signals containing an even number of carbons. The AHL signals also contain either oxo or hydroxyl modifications at the third carbon of the molecule. At this time, only one AHL synthesis gene, *afeI*, has been identified and characterized, but another open reading frame has been hypothesized to be an AHL synthase due to its sequence homology to the AHL synthase *hdtS* from *Pseudomonas fluorescens*.

AfeI is similar to LuxI of *V. fischeri* and produces five different AHLs when expressed in an AHL-lacking *Escherichia coli* strain. Immediately downstream of the *afeI* gene in *A. ferrooxidans* is the *luxR* homologue *afeR*. Transcriptional studies show that *afeI* and *afeR* transcripts are present and *afeI* transcript levels increase relative to increases in AHL concentration. Downstream regulatory targets for AfeR in *A. ferrooxidans* have not been fully determined, but it has been shown that *afeI* transcripts are strongly induced during phosphate-limiting conditions. It has also been shown that there are differential increases of specific AHL molecules during growth in sulfate, thiosulfate, or iron-containing medium. This data suggest that the type of AHL signal produced may be influenced by the growth substrate as well as cell density. The fact that *A. ferrooxidans* is often found in multispecies biofilms and produces a variety of AHL types could also suggest a role for *A. ferrooxidans* in modulating density-dependent expression of other species in response to growth substrates *in situ*.

The Study of AHL-Dependent Signal Pathways Demonstrated the Presence of Another Quorum Signal AI-2

V. harveyi and the LuxS-Produced Signal AI-2

Previously we discussed the discovery of quorum signaling in the luminescent marine bacteria *V. fischeri*. Further work with *V. fischeri* identified and characterized the LuxI/R regulatory circuit and the AI-1 HSL signal. However, determining the nature of the density-dependent signal in the closely related luminescent bacterium *V. harveyi* yielded different results. *V. harveyi* luminescence is density-dependent, inducible by spent culture medium, and is dependent upon a 3OHC_4-HSL. Despite these similarities, genes homologous to *luxI* and *luxR* were not found on the *V. harveyi* chromosome.

Mutational analysis determined that *V. harveyi* 3OHC_4-HSL was produced by the AHL synthase proteins LuxL and LuxM. It is not clear which protein is the actual synthase, but the presence of both is necessary for 3OHC_4-HSL production. Neither gene has significant sequence homology to *V. fischeri luxI* but appear to carry out similar reactions to synthesize 3OH-C_4-HSL. It was also found that a *luxN* gene encoded for a sensor kinase protein, which was necessary to sense extracellular AHLs. This mechanism is reminiscent of Gram-positive AIP sensors.

LuxN was later shown to autophosphorylate at low 3OHC$_4$-HSL concentrations and transfer phosphate to the LuxO response regulator via the intermediate LuxU. LuxO-phosphate represses expression of the *luxCDABE* luminescence operon, ensuring that light production is turned off at low AHL concentrations. Knockout mutants in either the *luxLM* synthesis genes or the *luxN* sensor kinase gene did not completely abrogate density-dependent expression of luminescence genes. This suggested the presence of a second quorum signaling system in *V. harveyi*.

The same mutational study that characterized the *luxL*, *luxM*, and *luxN* genes also showed that deletions in the sensor kinase *luxQ* and periplasmic binding protein *luxP* were necessary for density-dependent luminescence independent of the 3OHC$_4$-HSL signal. These proteins were shown to be part of a second sensory mechanism in *V. harveyi*. Reporter strains were created using the *luxP* and *luxQ* genes to detect a second signal. Initial biochemical characterizations revealed that a second signal could activate the *luxQP* reporter strains, and this signal was named AI-2. Surprisingly, AI-2 is produced by several other species of bacteria and the *luxS* gene responsible for AI-2 synthesis was identified in *V. harveyi*, *E. coli*, and *Salmonella typhimurium* shortly after.

LuxS synthesizes AI-2 as part of a detoxification reaction in the activated methyl cycle. Methyl transfer reactions are vital in bacterial metabolism and are dependent on the methyl donor SAM. SAM is converted into the toxic *S*-adenosylhomocysteine (SAH) molecule and can be detoxified by two different mechanisms. The first mechanism utilizes the SAH hydrolase gene, which hydrolyzes SAH into the SAM precursors adenosine and homocysteine. Organisms that do not produce AI-2, such as *P. aeruginosa*, utilize this pathway. The second mechanism utilizes the Pfs enzyme, which hydrolyzes SAH into adenosine and *S*-ribosylhomocysteine (SRH). Homocysteine is removed from SRH leaving 4,5-dihydroxy-2,3-pentanedione (DPD) in a reaction catalyzed by the LuxS enzyme; DPD spontaneously cyclizes to form the furanosyl diester AI-2. Recent work has shown that AI-2 in *V. harveyi* incorporates boric acid into its structure, the first known biological use of this molecule. To date, it does not appear that boron-containing AI-2 signals are used outside of the *Vibrio* genus and that AI-2 molecules lacking boron function as signals in other bacteria.

As mentioned above, AI-2 is recognized by the sensor kinase LuxQ via the periplasmic AI-2-binding protein LuxP. When a threshold concentration of AI-2 is reached, LuxQ changes from a kinase to a phosphatase, removing the phosphate from its downstream target LuxU. As LuxU is dephosphorylated, it removes phosphate from the LuxO protein. Unphosphorylated LuxO loses the ability to repress the *luxCDABE* operon, and luminescence genes are then activated by the transcriptional activator LuxR, which has no homology to the *V. fischeri* LuxR regulator. Thus, the *V. harveyi* quorum-signaling pathway utilizes the input of two separate density-dependent signals that act on the central regulator LuxO. This pathway is similar to the *Vibrio cholerae* quorum-signaling pathway described in **Figure 7**.

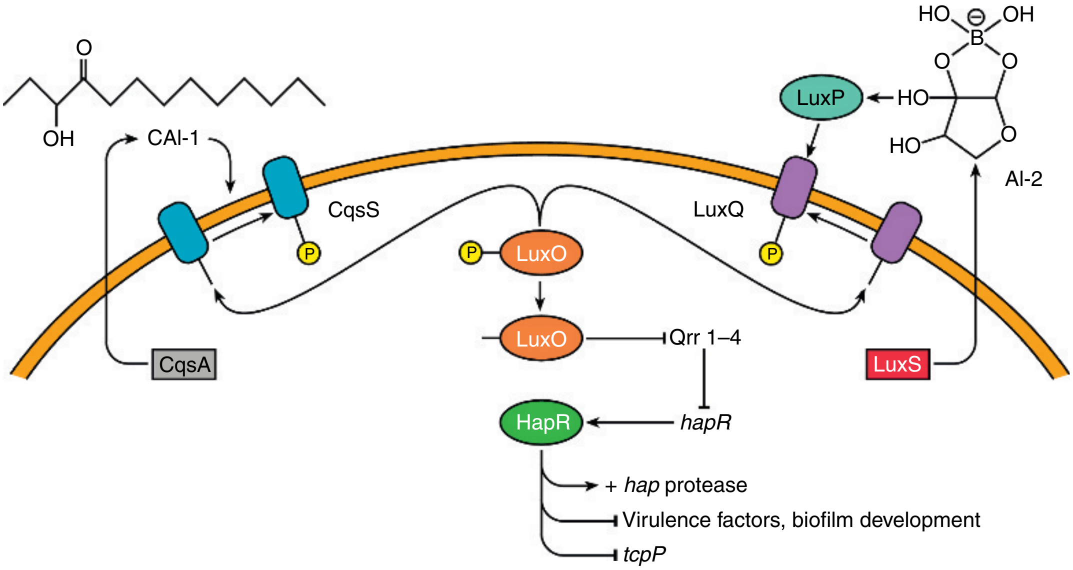

Figure 7 Quorum sensing in *Vibrio cholerae*. CqsA and LuxS produce CAI-1 and AI-2, respectively. CAI-1 interacts with the CqsS sensor phosphatase, and AI-2 interacts with the LuxQ sensor phosphatase via the AI-2-binding protein LuxP. Both phosphatases remove phosphate from the LuxO protein. Dephosphorylated LuxO is inactive and no longer induces expression of the small RNAs Qrr1-4, which served to repress *hapR* expression. Translation of HapR induces expression of the *hapR* protease gene and represses expression of genes involved in virulence and biofilm formation.

It is notable that AI-2 synthesis is directly linked to a key metabolic pathway in organisms that carry the *luxS* gene. Because of this link, it is hypothesized that LuxS production is an indicator of the metabolic status of the organism as well as its population density. This could be similar to the differential AI-1-type signal production seen in *A. ferrooxidans*. It should also be mentioned that only two genomes sequenced to date contain genes for both SAH detoxification mechanisms, and this phenomenon may indicate a point of divergence in bacterial evolution.

AI-2 and *V. cholerae*

V. cholerae is a free-swimming marine bacterium and is the causative agent of cholera in humans. *V. cholerae* is commonly found attached to the surface of zooplankton in the ocean. Cholera outbreaks can be associated with zooplankton blooms near human populations. During the course of disease, *V. cholerae* is ingested and survives the low pH of the stomach to colonize the host small intestine. During colonization, *V. cholerae* uses motility and mucinase to penetrate the mucus layer of the intestine and gain access to the underlying epithelial cell layer.

Currently, *V. cholerae* strain El Tor is the cause of a global cholera pandemic in underdeveloped nations. *V. cholerae* El Tor contains the HapR regulator that represses expression of virulence factors such as the cholera toxin (CT) and the cholera toxin coregulated pilus (TCP). TCP and CT have been shown to be critical for *V. cholerae* host colonization in animal models. The HapR regulator is acted upon by quorum signals in *V. cholerae* El Tor and links the quorum signaling pathway to virulence factor production. Previous pandemic strains of *V. cholerae* contain mutations within the *hapR* gene and are not considered to be responsive to quorum signaling.

V. cholerae contains a quorum sensing system that is similar to *V. harveyi*. However, one of the autoinducer signals as well as the genes regulated by quorum signaling are different in *V. cholerae*. In *V. cholerae*, the AI-2 signal and the recently characterized signal (*S*)-3-hydroxytridecane-4-one (CAI-1) are recognized by the sensor kinases LuxQ and CqsS, respectively (**Figure 7**). In low cell density conditions, LuxQ and CqsS act as kinases that phosphorylate LuxU, which then transfers its phosphate to LuxO much like the pathway in *V. harveyi*. Phosphorylated LuxO induces the expression of four small regulatory RNAs (sRNAs) known as Qrr1-4. These sRNAs interact with the sRNA chaperone protein Hfq and induce expression of an uncharacterized regulatory gene (vca0939) as well as destabilize the mRNA for *hapR*, the master quorum signaling regulator. When *hapR* is repressed, virulence genes are upregulated, due in part to removal of HapR repression of the *tcpP* gene, which encodes the signaling protein TcpP that induces expression of the ToxT regulator. ToxT induces expression of the virulence factors TCP and CT, so in conditions of low cell density and autoinducer concentrations, virulence and biofilm development genes are upregulated. TCP allows for cell–cell aggregation of *V. cholerae* as well as attachment to the host. CT activity causes epithelial cells to excrete large amounts of water, ultimately causing the host to rapidly expel its intestinal contents, one of the hallmark symptoms of a *V. cholerae* infection.

At high cell densities, LuxQ and CqsS act as phosphatase in the same fashion as LuxQ and LuxN in *V. harveyi*. LuxQ and CqsS ultimately cause the dephosphorylation of LuxO, which causes expression of the Qrr sRNAs to be decreased. As Qrr sRNA expression decreases, *hapR* is upregulated. HapR blocks expression of virulence factors and genes involved in biofilm development. HapR also induces expression of the *hap* gene, which encodes for a protease that aids *V. cholera* in release from a colonized area. In *V. cholerae*, density-dependent signals provide the cell with a method to change cell behavior from a colonizing, scavenging lifestyle at low density, to a motile, migratory lifestyle at high cell density. Interestingly, this change in behavior occurs at a time when pathogenic effects of *V. cholerae* are inducing the host to expel large amounts of intestinal contents, thus allowing the freshly liberated *V. cholerae* to return to a free-swimming lifestyle. It is also interesting that quorum signal-utilizing pathogens such as *S. aureus* and *P. aeruginosa* upregulate virulence factors at high density to form persistent infections, whereas *V. cholerae* downregulates virulence and rapidly leaves the host once high population density has been achieved.

E. coli, *S. typhimurium*, and AI-2

The Gram-negative enteric bacteria *E. coli* and *S. typhimurium* both grow in the lower intestine of mammals. While certain *E. coli* strains are pathogenic, many strains are part of the normal flora. *S. typhimurium* is the causative agent of typhoid fever and, while closely related to *E. coli*, is often a more transient inhabitant of the gastrointestinal tract. Both of these bacteria produce AI-2 using the LuxS enzyme, with maximum production occurring at mid-exponential phase growth. Surprisingly, extracellular AI-2 concentrations drop rapidly as either organism enters stationary phase. This suggests a mechanism for AI-2 sequestration or degradation.

AI-2 has been shown to regulate several genes in both *E. coli* and *S. typhimurium*. These genes include the *lsrACDBFGE* operon, the *lsrK* kinase gene, and *lsrR* whose product represses the *lsr* operon. In both organisms, AI-2 is produced during growth and accumulates outside of the cell. When threshold AI-2 concentrations are reached, AI-2 is imported by the LsrABCD ABC transporter. After import into the cell, AI-2 is phosphorylated by the LsrK kinase and catabolized by the LsrE, LsrF, and LsrG proteins. The products of phospho-AI-2 catabolism have not been characterized at the time of this writing. While the import and catabolism of AI-2 by these organisms is regulated in a density-dependent fashion, it is unclear if these organisms utilize AI-2 to direct group activities. The simplest explanation is that AI-2 is produced as a metabolite during SAH detoxification in the activated methyl cycle and is taken as backup to be utilized as a carbon source later in the growth phase.

Despite the possibility that *E. coli* and *S. typhimurium* may utilize AI-2 only as a metabolite, production of AI-2 by these bacteria may affect gene expression of other AI-2 responsive bacteria in polymicrobial environments. In a 2005 *Nature* paper, Karina Xavier and Bonnie Bassler demonstrated that when *V. harveyi* or *V. cholera* were grown in coculture with *E. coli*, they were subject to interference of AI-2-mediated signaling due to the ability of *E. coli* to respond to higher AI-2 concentrations. As *E. coli* cell density increased, it was able to remove AI-2 from the culture and reduce expression of AI-2-dependent genes in either *Vibrio* species. At low cell densities, premature induction of AI-2-regulated genes was observed in *V. harveyi*, as the AI-2 produced by *E. coli* increased the AI-2 concentration that *V. harveyi* would normally encounter at that cell density. It is apparent from these studies that AI-2 signal turnover can have substantial effects on multispecies interaction even if AI-2 does not appear to have a strong role in quorum-signal-dependent behavior in a single species.

luxS-Dependent Interactions in Human Oral Bacteria

The human oral cavity is populated by over 300 different bacterial species. There are several distinct niches within the oral cavity (tooth enamel surface, epithelial cell surface, and the subgingival space), all of which can be colonized by bacteria growing in multispecies biofilms. Many species isolated from the oral cavity contain the *luxS* gene, and in several cases, density-dependent AI-2-mediated behavior has been observed in single and multispecies cultures.

Streptococcus mutans is a common inhabitant of the oral cavity and is the causative agent of dental caries. Merritt and Shi in a 2003 *Infection and Immunity* paper demonstrated that biofilm formation by *S. mutans* was altered in a *luxS* mutant. The *S. mutans luxS* mutant formed a biofilm with denser cell aggregates that was more resistant to detergent and antibiotics. While growth rate and acid production did not appear to be altered in the *luxS* mutant, it was not determined whether extracellular AI-2 concentration or the effects of impeded SAH detoxification were responsible for the mutant phenotype.

Direct AI-2-dependent interaction in oral bacteria was reported in 2003 by McNab and Lamont in the *Journal of Bacteriology*. The researchers demonstrated that *Streptococcus gordonii* and *Porphyromonas gingivalis* formed significantly dense biofilm structures if either organism contained a functional *luxS* gene. Simultaneous *luxS* mutations in both species led to diminished biofilm formation, suggesting that *luxS*-dependent AI-2 formation from one organism can complement the ability of the other to form more fully developed multispecies biofilms.

This phenomenon is similar to another described in a 2004 *Proceedings of the National Academy of Sciences* paper by Egland and Kolenbrander. In that study, *S. gordonii* was shown to upregulate production of amylase only when grown in close proximity to *Veillonella atypica*. This interaction was hypothesized to be dependent on an increase in local concentration of a diffusible signal when the bacteria grow in close proximity to one another. This data suggest that signal production by *S. gordonii* alone is insufficient to upregulate amylase under the conditions tested, and the presence of another signal-producing organism is also necessary. AI-2 is hypothesized to be the diffusible signal in this system, but this has not been unequivocally shown. Both of these cases demonstrate that extracellular signal concentrations are critical to modulate behavior in each organism. This is notable because in the case of *S. mutans* and other literature discussing *luxS*-dependent signaling events, it is often unclear if the phenotypes observed are due to actual AI-2-dependent signaling, or due to metabolic effects caused by an impediment of SAH detoxification.

AI-2-dependent quorum signaling has also been observed in the opportunistic pathogen *Aggregatibacter actinomycetemcomitans*. LuxS activity or exogenous AI-2 addition to cultures have been shown to cause differential expression of iron uptake genes as well as leukotoxin production. AI-2 production by *A. actinomycetemcomitans* has also been shown to complement *luxS*-dependent gene expression in a *P. gingivalis luxS* mutant when cocultured. A 2007 *Infection and Immunity* paper by Shao and Demuth demonstrated that *A. actinomycetemcomitans* is impaired for biofilm production in a *luxS* mutant. Subsequent complementation of *luxS* by *P. aeruginosa sahH* seems to restore the ability to detoxify SAH in the activated methyl cycle, but exogenously added AI-2 was necessary to restore the biofilm phenotype. While *sahH* transcripts were present in the complemented strain, it is not clear if SahH was produced and able to detoxify SAH. Despite the unknown status of SahH activity, this data strongly suggest that AI-2 serves as a signal for community behavior in *A. actinomycetemcomitans*, and not just a metabolite produced in the activated methyl cycle.

AI-2 signaling is responsible for density-dependent regulation of genes in several well-characterized systems. At this time, over 153 separate eubacterial genomes contain a *luxS*-like sequence according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. It is evident that in the case of *Vibrio* species and several inhabitants of the mammalian oral cavity, AI-2-dependent signaling plays a critical part of these organisms' lifestyle. However in many organisms, the presence of a *luxS* gene does not correlate to density-dependent signaling behavior. The 'other' function of LuxS as part of the activated methyl cycle is an important component of metabolism, and it cannot be assumed at this time that phenotypic changes in *luxS* knockouts are exclusively due to AI-2 signaling and not disruption of SAH detoxification.

Other Types of Quorum Signals

In addition to AI-1, AI-2, and peptide autoinducer signals, there are many other types of quorum signals utilized by bacteria. We are only beginning to understand the molecular aspects of these systems, and in some cases the structure of the signal is not known.

The AI-3/Epinephrine Quorum Signaling System

E. coli is a common inhabitant of the human lower intestine. Recently, strains have evolved that carry multiple virulence factors giving rise to enterohemorrhagic *E. coli* (EHEC) strains such as *E. coli* O157:H7. EHEC strains are notorious for their ability to cause hemorrhagic colitis and hemolytic-uremic syndrome. As EHEC colonizes the lower intestine, it forms lesions on host epithelial tissue and produces a Shiga-type toxin. A majority of the genes involved in attachment, lesion formation, and virulence exist on the EHEC chromosome in a pathogenicity island known as the locus of enterocyte effacement (LEE).

Expression of *LEE* genes is regulated by factors present in the native *E. coli* chromosome as well as factors encoded on LEE itself. In addition to these, some *LEE* genes appear to be regulated in a cell density-dependent fashion. Preliminary studies of a *luxS* EHEC mutant showed changes in LEE gene expression, indicating that AI-2 may play a role in regulation. However, the addition of exogenous AI-2 to the *luxS* mutant did not complement LEE gene regulation. This data suggested that LEE gene regulation was subject to metabolic changes observed in the *luxS* mutant. It was then shown that LEE gene regulation could be complemented by concentrated extracts from *luxS* mutant supernatants, and that an unidentified compound chemically different from AI-2 was synthesized at a low rate in the *luxS* mutant. LEE gene expression phenotypes were also restored in a *luxS* mutant strain that was complemented with the SAH hydrolase enzyme from *P. aeruginosa*. This experiment determined that homocysteine formation by either the SAH hydrolase enzyme or LuxS restored LEE gene expression. This suggested the presence of an inducer deemed, AI-3, whose biosynthesis utilizes precursors from the activated methyl cycle.

Other studies indicated that LEE gene expression could be influenced by epinephrine and norepinephrine hormones from the host, and it was hypothesized that AI-3 might resemble epinephrine/norepinephrine due to similarities in chemical properties. At this time, the structure of AI-3 is still unclear; however, the addition of exogenous epinephrine/norepinephrine induces similar sets of genes when compared to addition of impure AI-3 extracts. It has also been shown that AI-3 and epinephrine induce *LEE* genes as well as other targets and are dependent on the sensor histidine kinase QseC. EHEC are likely to come in contact with epinephrine/norepinephrine in the lower intestine during colonization as norepinephrine is produced by neurons in the enteric nervous system and epinephrine is secreted as a systemic response to host stress during EHEC disease progression. The discovery of QseC as a receptor for both a host-produced hormone and a bacterially produced quorum signal is astounding and contains many implications for future discoveries in quorum signaling as well as evolution between the bacterium and the host.

Bradyrhizobium japonicum Bradyoxetin Signal

Bradyoxetin is produced by the nitrogen-fixing soil bacterium *B. japonicum*. Genes involved in biosynthesis are unknown at this time, although the biosynthetic pathway is likely similar to that of the siderophore mugenic acid. Similar to siderophores, bradyoxetin production is regulated by Fe^{3+} concentration as well as cell density with bradyoxetin produced maximally at high cell densities or at low Fe^{3+} concentrations. At high cell densities, bradyoxetin appears to upregulate its own synthesis as well as the response regulators *nolA* and *nodD2*, whose products repress the expression of *nod* genes necessary for symbiotic root nodule formation in legumes. This system is yet another instance of quorum signal production being controlled by population density as well as the metabolic state of the organism.

Pseudomonas aeruginosa Pseudomonas Quinolone Signal (PQS)

In addition to the LasI- and RhlI-produced AI-1 AHL signals, *P. aeruginosa* synthesizes a quinolone compound PQS important for quorum signaling gene expression. PQS was discovered in 1999 by Everett Pesci, and its chemical structure is 2-heptyl-3-hydroxy-4-quinolone (**Figure 5**). PQS is synthesized from anthranilate via the *phnAB* and *pqsABCDE* gene products, which produce the precursor 4-hydroxy-2-heptyl-quinoline (HHQ). HHQ is converted into PQS by the PqsH protein.

PQS functions as part of the AI-1-mediated quorum signaling network in *P. aeruginosa*. Activated LasR (bound to 3OC$_{12}$-HSL) can upregulate *pqsH*, ultimately increasing PQS concentration in a 3OC$_{12}$-HSL-dependent manner. As PQS concentration increases, it induces *rhlI* expression in a *rhlR*-dependent manner. PQS negative mutants lack the ability to produce many virulence factors that are also absent in *rhlR* mutants. Thus, PQS serves as a link between the LasI/R and RhlI/R quorum signaling systems (**Figure 5**).

PQS is also interesting due to its hydrophobic nature. The hydrophobicity of PQS suggests that it would be a poorly diffusible signal in aqueous environments. Recently, it has been discovered by our lab that PQS is transported by outer membrane vesicles. This phenomenon allows PQS to be moved by a water-soluble carrier that may also serve to protect the signal from extracellular degradation by other organisms. Another interesting property of PQS is that its production appears to be restricted to *P. aeruginosa* (at least to this point), while its precursor HHQ is synthesized by many other Gram-negative bacteria, notably *Burkholderia*, *Pseudomonas*, and *Alteromonas* species. HHQ has also been shown to act as a signal in *P. aeruginosa* because of its release and uptake by neighboring cells and subsequent conversion into PQS. Thus, the production of 2-alkyl-4-quinolone compounds by these organisms suggests another class of quorum signaling molecules.

Ralstonia solanacearum 3-Hydroxypalmitic Acid Methyl Ester (3-OH PAME)

R. solanacearum is a Gram-negative plant pathogen that causes wilting in many different plant species. *R. solanacearum* infects plants at the root and over several days enters the xylem where it travels to the aerial portions of the plant. *R. solanacearum* colonizes the xylem and expresses several extracellular and intracellular virulence factors. A primary virulence factor in wilting disease is the production of copious amounts of EPS that physically blocks fluid transport through the xylem of the plant, thereby leading to wilting. This disease is very similar to the previously mentioned Stewart's wilt disease caused by *P. stewartii*.

R. solanacearum virulence factor production is ultimately controlled by the concentration of the quorum signal 3-OH PAME (**Figure 8**). 3-OH PAME is produced by the PhcB protein and released from the cell. When 3-OH PAME reaches a threshold concentration, it interacts with the sensor kinase PhcS, which then transfers a phosphate group to inactive the PhcR protein. PhcR inactivation allows the regulator PhcA to activate. PhcA ultimately controls the expression of many virulence genes in *R. solanacearum* as well as a LuxI/R like signaling system encoded by the *solI/R* genes.

3-OH PAME is significant among quorum signals because it is a volatile compound. Volatility of a quorum signal has positive and negative consequences in that it allows rapid signaling over greater distances than an aqueous signal; however, the signal can also diffuse away from receptive cell populations before a threshold

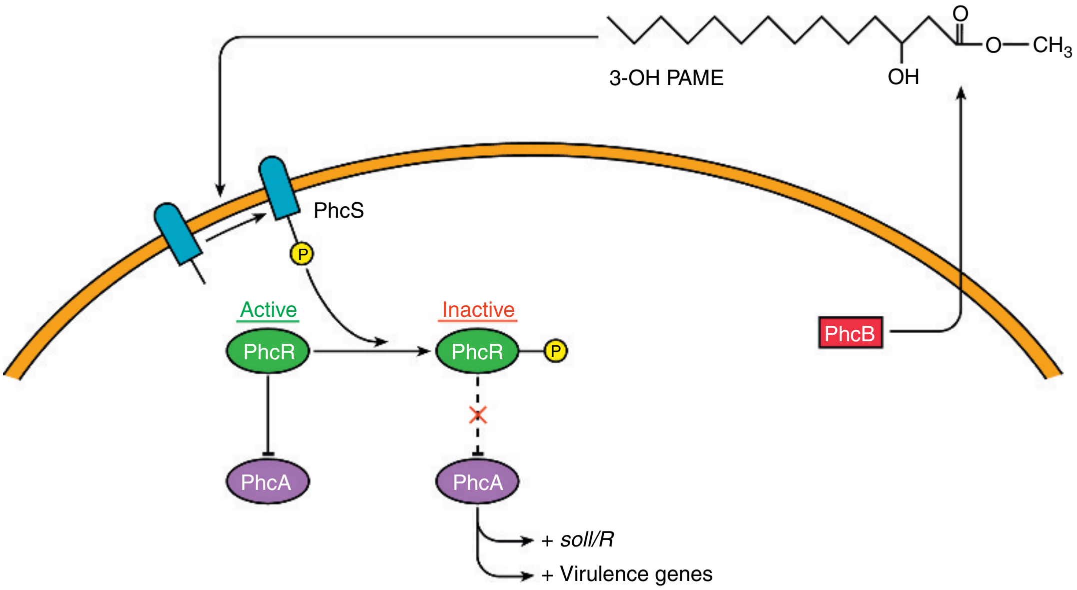

Figure 8 Quorum sensing in *Ralstonia solanacearum*. PhcB synthesizes the volatile signal 3-OH PAME. 3-OH PAME interacts with the PhcS sensor kinase to phosphorylate the PhcR regulatory protein. PhcR-phosphate is unable to block activity of the PhcA transcriptional regulator that induces expression of virulence genes as well as the *solI/R*-dependent AHL quorum sensing system.

concentration is reached. It is interesting that a volatile signal appears to function sufficiently inside plant tissues, which are highly heterogeneous in terms of fluid and gas density. It is also notable that *R. solanacearum* appears to use multiple quorum signal cascades to regulate virulence gene expression much like *P. aeruginosa.*

Extracellular Effectors of Quorum Signaling Systems

Quorum signals are produced by many diverse bacterial species in many different environments. The ability to utilize signals to direct efficient and timely changes in gene expression almost certainly provides that species with a strong competitive advantage for nutrient acquisition and defense. Likewise, a competitor or host would gain tremendous benefit if it were able to interfere with a pathogen or competitors' quorum-sensing system. The evolutionary 'arms race' between quorum signaling and signal interference by other species could be millions of years old, yet was just recently revealed by researchers.

In 1996, Michael Givskov and Staffan Kjelleberg and colleagues described the production of AHL mimics by the marine seaweed *Delisea pulchra. D. pulchra* produces several halogenated furanone molecules that structurally resemble AHL signals. Furanones were shown to inhibit quorum signal-dependent swarming motility of *Serratia liquefaciens* as well as several marine bacteria. AHL reporter systems in *E. coli* as well as *V. harveyi* were shown to be inhibited by exogenous addition of furanones. Further work on *D. pulchra* furanones showed that they disrupt quorum signaling by binding to LuxR-type proteins in a competitive fashion with the native AHL signal. In the case of *D. pulchra*, furanone-dependent quorum inhibition could protect the plant from colonization by pathogenic bacteria that regulate motility, biofilm, or virulence gene expression through quorum-signaling systems.

A similar phenomenon was observed in the unicellular eukaryotic alga *Chlamydomonas reinhardtii*, which also produces quorum signal mimics. *C. reinhardtii* grows in soil and freshwater environments where it likely encounters many bacterial species that utilize quorum signaling. It was shown that *C. reinhardtii* produced at least 12 uncharacterized compounds that stimulated the LasR and CepR regulators in *E. coli* reporter strains. Interestingly, none of the compounds stimulated LuxR or AhyR regulators in similar reporter strains, suggesting that the putative AHL mimics produced by *C. reinhardtii* are receptor-specific. The chemical properties of the *C. reinhardtii*-produced mimics suggest that they are AHL-like in nature, but subsequent characterization of the compounds revealed that they are not known AHLs. Despite their dissimilar structure, *C. reinhardtii* AHL mimics were shown to increase the production of many AHL-controlled genes to levels observed with the native AHL; however, some proteins were found to be downregulated by the AHL mimics. The full impact of these results is still unclear, but demonstrates that *C. reinhardtii* likely has significant effects on bacterial quorum signaling in the natural environment.

Recent research by several groups has identified eubacterial and eukaryotic enzymes that degrade AHLs. There are many incidences of production of these enzymes in bacteria, and it appears that one function may be to degrade the bacteria's own AHL signal in order to downregulate the quorum signal response after its desired effects are achieved. Another function of these enzymes may be to degrade AHLs from competing organisms as a means of interfering with their quorum signaling systems. The observation that some enzymes produced by AHL-synthesizing bacteria cannot degrade their own AHLs, yet degrade those produced by other organisms, supports the idea of competitive interference between species.

In a 2004 *Proceedings of the National Academy of Sciences* paper, Carlene Chun and E. Peter Greenberg demonstrated that human airway epithelial cells were able to degrade the $3OC_{12}$-HSL from *P. aeruginosa.* As mentioned previously, *P. aeruginosa* can cause respiratory infections in humans. During the course of infection, airway epithelial cells are likely to come in contact with colonizing *P. aeruginosa,* and the ability of the host to interfere with quorum signaling may be of great importance in preventing infection. Surprisingly, this study showed that the degradation of $3OC_{12}$-HSL was contact-dependent and that degradation did not occur in culture supernatants from the airway cells. It was also shown that degradation was specific for $3OC_{12}$-HSL, C_{12}-HSL, and C_6-HSL. No degradation of C_4-HSL or $3OC_6$-HSL was observed. The AHL degradation phenotype varied widely among cell types tested but seemed to occur most frequently in cell lines that were derived from epithelial cells that are most likely to encounter AHL-producing organisms. These data suggest that humans, and potentially other mammals, have evolved mechanisms to specifically defend against AHL-producing bacteria.

Quorum Signal Interaction with the Host

Eukaryotic hosts utilize both quorum signal-degrading enzymes and mimics to interfere with bacterial quorum signaling. Some organisms produce these substances throughout their life cycle, while others produce them in response to bacterial quorum signals. The ability of eukaryotic organisms to sense and respond to quorum signals is fascinating and further emphasizes the affect that prokaryotes may have on the evolution of eukaryotes and vice versa. We will describe a few representatives from the growing number of cases of eukaryotic responses to bacterially produced quorum signals.

The legume *Medicago truncatula* senses and responds to AHL signals produced by *Sinorhizobium meliloti* and *P. aeruginosa.* Many *M. truncatula* proteins are regulated in a similar manner when exposed to AHLs from either bacterial species; however, some proteins are differentially expressed in response to AHLs from one species compared to the other. This suggests that *M. truncatula* has a conserved response to AHLs but also has the ability to discriminate between AHL signals from these bacteria. AHL signals were found to differentially regulate over 150 proteins in *M. truncatula*, the most notable of which were involved in primary metabolic pathways, stress response, and enzymes used in quorum signal mimic production. At this time, it is unclear how these responses specifically benefit *M. truncatula* or how they affect *S. meliloti* or *P. aeruginosa,* but it is apparent that both organisms stimulate a significant change in behavior of the plant.

In 2006, Elmus Beale and Kendra Rumbaugh demonstrated that the soil nematode *Caenorhabditis elegans* is able to sense AHL signals from bacteria. *C. elegans* was shown to preferentially move toward AHL-producing organisms as opposed to AHL-lacking species. This AHL-dependent chemotaxis is thought to aid *C. elegans* in locating bacteria as a source of food. Another surprising observation was that *C. elegans* would move toward *P. aeruginosa* despite the fact that *P. aeruginosa* kills *C. elegans*. After exposure to *P. aeruginosa*, surviving *C. elegans* would display avoidant behavior toward AHL-producing organisms including nonharmful species. This work provides an elegant example of a eukaryote behavioral response to bacterial quorum signals. Another study by the same lab described the ability of *P. aeruginosa* AHLs to enter and modulate expression in mammalian cells; thus demonstrating that AHLs are able to cross the host cell membrane and stimulate host gene expression. These changes in gene expression are thought to occur through AHL stimulation of host nuclear hormone receptors. Further work demonstrated that *P. aeruginosa* AHLs can induce inflammation, immunosupression, and apoptosis in immortalized cells as well as primary cell cultures. Many of these events are hallmarks of tissue exposed to *P. aeruginosa* during infection, but it remains to be determined if the responses seen during infection are actually due to *in vivo* AHL signaling or the myriad of other virulence factors *P. aeruginosa* produces.

Diffusion Sensing, Efficiency Sensing, and the Future of Quorum Signaling Systems

In 2002, Rosemary Redfield at the University of British Columbia proposed that quorum sensing may be a property of what she referred to as diffusion sensing. Before her proposal of this concept, quorum signaling had been widely accepted as a cell density-dependent signal that affected gene expression of a population, with the consequence of directing a population to exhibit behavior that affected the group as a whole. The concept of diffusion sensing proposes that individual bacteria sense autoinducer concentrations and subsequent changes in gene expression direct a response that is intended for the individual cell, not the population as a whole. She proposed that evolutionary selection for quorum sensing behavior was selected for by the benefit given to an individual cell that has the ability to use a signal to gather information on properties of the local environment.

While the concept of diffusion sensing is interesting, it must be considered that any receptor-based system should be induced when ligand concentration exceeds that of the receptors' affinity for it. This condition can be achieved either by increased production of signal, decreased mass transfer rate, or decreased local volume around the cell. In quorum signaling, we ascribe that signal-dependent behavior is due to an increase in population and subsequent increase in signal. In many instances of quorum signaling systems, it may indeed be a product of diffusion sensing by the cell. However, observations of autoinducer-producing organisms in some characterized systems seem to indicate behavioral changes that are more beneficial to a group of cells as opposed to an individual. These changes include density-dependent luminescence as well as conjugation, which serve little purpose when local cell numbers are low.

Hense *et al.* in a 2007 *Nature* review attempted to unify the gap between diffusion and quorum sensing and propose the novel concept of efficiency sensing. Efficiency sensing suggests that cells produce signaling compounds to 'test' the population density, volume, and mass transfer rate of their environment. If signal concentration increases, then induction of energetically expensive, signal-dependent pathways occurs. This hypothesis takes into account that signal concentration can be affected by local concentration of cell density as well as the mass transfer rates and the volume of the local environment. Hense and colleagues propose that efficiency sensing provides a means for individual as well as community advantages of diffusible signals to overlap and provide evidence through mathematical models that indicate that spatial distribution of cells may be more important than density.

It is possible that diffusion sensing behavior in individuals may be the primary means of positive selection for organisms that have quorum signaling systems of either low complexity or are newer aspects in the organism's evolutionary history. Thus as bacteria adapt and evolve, diffusion sensing behavior can be recruited into true quorum signaling behavior and exert a community benefit on a population of cells.

What's Next for Quorum Signaling?

Research in quorum signaling initially uncovered a few specific cases that demonstrated density-dependent signaling and its impact on a single response in the signaling organism. Phenomena such as luminescence and regulation of competence were largely thought to be the single process in the cell directed by a diffusible signal, and much initial research focused on utilization of a specific signal and its interaction with a single receptor or transcriptional regulator to direct regulation of just a few related genes or operons. The discovery of orphan regulators in *P. aeruginosa* and other species and the convergence of multiple types of signals involved in gene expression in *V. harveyi*, *V. cholerae*, *P. aeruginosa*, *R. solanacearum*, and *B. subtilis* (to name a few) demonstrate that many organisms utilize quorum signals in increasingly complex pathways. Further research into orphan regulators and how bacteria integrate multiple quorum signal pathways may reveal an even greater role for quorum signaling in bacteria than was previously considered.

There are ever increasing observations of interspecies quorum signal interactions between bacteria as well as interdomain interactions between bacteria and eukaryotic hosts. These studies demonstrate that prokaryotes and eukaryotes may utilize signals not only as a means to detect signal producers, but as a way to defend against them *in situ*. These adaptations shed light on possible therapeutic strategies for infections caused by quorum signal-producing bacteria. The discovery of quorum signal interference, especially in plants, may be useful in developing new variants of commercial crops that are more resistant to bacterial infections.

A poorly understood aspect of quorum signaling is that some microorganisms utilize quorum signals not only as a strict density-dependent signal but also as a way of monitoring and reporting the nutrient status of the environment. PQS signals in *P. aeruginosa* have been shown to be differentially produced in different environmental concentrations of aromatic amino acids. Also, the types of quorum signals produced by *A. ferrooxidans* vary depending on the substrates available to the organism. These phenomena as well as the production of AI-2 and its dependency on the metabolic status of the cell indicate that quorum signal production may serve functions aside from monitoring cell density.

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Sensory Transduction in Bacteria

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Defining Statement
General Principles of Signal Transduction
Types of Bacterial Receptors and Signaling Pathways
Structural Organization of Signal Transduction Proteins
Interaction of Signal Transduction Pathways
How *E. coli* Sees the World?
Further Reading

Glossary

EAL domain Conserved protein domain with c-di-GMP-specific phosphodiesterase activity, named after its conserved Glu-Ala-Leu sequence motif.

GAF domain Conserved ligand-binding protein domain, named after cGMP phosphodiesterases, adenylyl cyclases, and FhlA protein, where it was originally described.

GGDEF domain Conserved protein domain with diguanylate cyclase (c-di-GMP synthetase) activity, named after its conserved Gly-Gly-Asp/Glu-Glu-Phe sequence motif.

HAMP domain Conserved α-helical cytoplasmic linker domain, named after histidine kinases, adenylyl cyclases, and methyl carrier proteins, where it was originally described.

HD-GYP domain Conserved protein domain with c-di-GMP-specific phosphodiesterase activity, named after its two conserved sequence motifs, His-Asp and Gly-Tyr-Phe.

PAS domain Conserved ligand-binding protein domain, named after period circadian protein (Per), aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded protein (Sim) proteins, where it was originally described.

phosphorylation Transfer of the phosphoryl group $-PO_3^{2-}$, for example, from ATP to various acceptors.

protein domain A discrete structural unit of proteins that can be found in different contexts. Most domains range in size from 25 to 300 amino acid residues.

protein sequence motif A group of amino acid residues, conserved among several different proteins.

REC domain Conserved protein domain that is similar to the CheY chemotaxis protein and contains a conserved Asp residue that can be reversibly phosphorylated by histidine kinases or artificial phosphoryl donors.

Abbreviations

cAMP	cyclic adenosine monophosphate
c-di-GMP	cyclic dimeric bis-(3′-5′)-guanosine monophosphate
CDD	Conserved Domain Database
Dos	direct oxygen sensor
EI	enzyme I
EIIA^{Glc}	enzyme IIA^{Glc}
EIIA	enzyme IIA
EIIB	enzyme IIB
HPt	histidine phosphotransfer
HTH	helix-turn-helix
InterPro	Integrated database of protein families, domains and functional sites
KEGG	Kyoto Encyclopedia of Genes and Genomes
KinG	Kinases in Genomes
MCPs	methyl-accepting chemotaxis proteins
MiST	Microbial Signal Transduction
PEP	phosphoenolpyruvate
PTS	PEP-dependent sugar phosphotransferase system
ProDom	Protein Domains
REC	receiver
Sentra	Sensory Signal Transduction Proteins
SMART	Simple Modular Architecture Research Tool
wHTH	winged helix-turn-helix

Defining Statement

Bacterial signal transduction systems include several types of receptor proteins that monitor the conditions inside and outside the cell and control the cellular response to the changes in environmental parameters at the level of individual genes and proteins, the whole-cell level (chemotaxis), and the level of multicellular communication (biofilm formation).

General Principles of Signal Transduction

The ability to sense changes in the environment and respond to them by adjusting its internal organization is a key property of all living cells. In principle, the level of a cellular metabolite can be regulated by simple feedback mechanism with a single transcriptional regulator. For example, a simple system consisting of a cellobiose-binding transcriptional regulator and a cellobiose-degrading enzyme could be sufficient for controlling the level of cellobiose in the cell. Increased levels of cellobiose would shift a larger proportion of the transcriptional regulator into the active (DNA-binding) form, which would lead to increased expression of the cellobiose-degrading enzyme that would eventually lead to the decrease in cellobiose level. Decreased levels of cellobiose would cause dissociation of the sugar from the transcriptional regulator, gradual decrease in the expression of cellobiose-degrading enzyme, and, as a result, stabilization of the cellobiose level.

This simple schema allows the cell to respond to the changing levels of a cellular metabolite and is used – with various modifications – by a variety of regulatory systems. However, this kind of regulation is necessarily rather crude and would not allow coordinated regulation of complex metabolic pathways. Rather than measure the levels of nutrients in the cytoplasm, it is much more advantageous to monitor their levels in the environment. That would make it possible, even at relatively low levels of certain nutrients outside the cell, to induce the appropriate transport systems and bring these nutrients into the cell. This is exactly the strategy utilized by many cells. For example, uptake of glucose-6-phosphate and several other sugar phosphates by *Escherichia coli* cells can be induced by micromolar levels of extracellular glucose-6-phosphate, which are 1000 times less than the concentration of this glycolytic intermediate in a normally growing cell. As another example, sensing of antibiotics and other toxins should occur before (or at lower concentrations) these compounds can irreversibly damage the cell. The question then becomes how to detect an extracellular signal and transmit it inside the cell without confusing the extracellular metabolite with its intracellular pool. Summing up, bacterial signal transduction systems, as opposed to simple feedback mechanisms, are usually utilized to

- detect extracellular signals, particularly weak ones;
- detect antibiotics, toxins, and other hazardous compounds before they damage the cell; and
- regulate complex metabolic processes.

The complexity of cellular organization calls for coordinated response(s) to environmental challenges, making it necessary for the signal transduction machinery to

- monitor complex traits (pH, osmotic stress, and redox level);
- maintain hierarchical organization and proper interaction of various transcriptional units; and
- coordinate cellular responses on several different levels (e.g., couple transcriptional regulation with chemotaxis).

Detection of environmental signals is usually carried out by transmembrane proteins that contain an extracytoplasmic (periplasmic or extracellular) domain, usually located at the N-terminus of the protein, one or several transmembrane segments, and a cytoplasmic domain (**Figure 1**). The cytoplasmic domain almost always has an enzymatic activity, occupies the C-terminal part of the protein, and serves as the signal transmitting module. It is probably no coincidence that most of these cytoplasmic modules are enzymatically active in their dimeric form (see below). It is believed that signal reception (i.e., binding of some ligand) by the extracellular module causes a conformational change that favors dimerization of the whole protein and therefore stimulates the enzymatic activity of its cytoplasmic module. The mechanisms of the propagation of the signal across the membrane are still not clear and are subject of intensive research.

The environmental signals monitored by the bacterial cell vary in nature and intensity and may include changes in the temperature, pressure, light intensity, salt concentration, changes in the levels of certain nutrients in the surrounding medium, levels of oxygen and reactive oxygen species, levels of other terminal electron acceptors, CO_2, CO, NO, and other dissolved gases, presence of other cells nearby, and possibly many other parameters. Accordingly, the cellular responses can occur at the level of individual genes and operons (changes in gene expression), at the whole-cell level (chemotaxis, phototaxis, and sporulation), and at the level of multicellular communication (e.g., biofilm formation). The regulation of gene expression can occur at the level of transcription (changes in expression of certain genes, operons, or even global regulons), post-transcriptional (e.g., changes in the mRNA stability), and post-translational (e.g., modulation of enzyme activity) regulation. For this reason, the signal transduction machinery includes not just mechanisms of communication between the sensory and the response modules, but also these modules themselves.

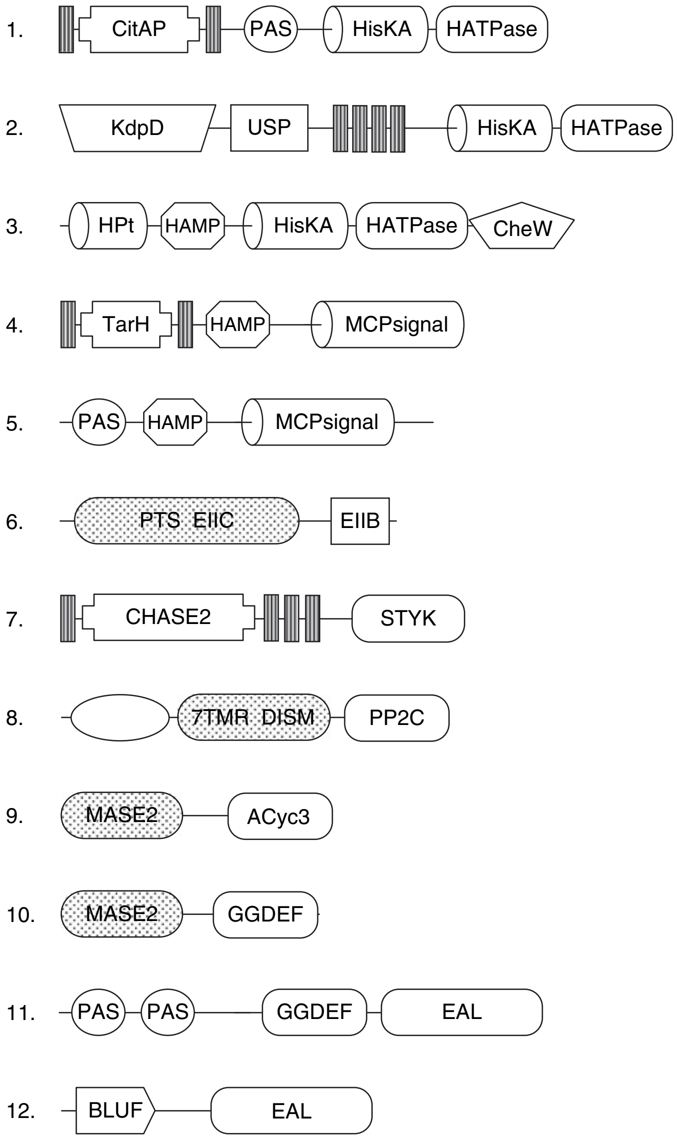

1.
CitAP
PAS
HisKA
HATPase
2.
KdpD
USP
HisKA
HATPase
3.
HPt
HAMP
HisKA
HATPase
CheW
4.
TarH
HAMP
MCPsignal
5.
PAS
HAMP
MCPsignal
6.
PTS_EIIC
EIIB
7.
CHASE2
STYK
8.
7TMR_DISM
PP2C
9.
MASE2
ACyc3
10.
MASE2
GGDEF
11.
PAS
PAS
GGDEF
EAL
12.
BLUF
EAL

Figure 1 Domain architectures of some bacterial receptors. 1 – *Escherichia coli* citrate-sensing histidine kinase DpiB (UniPROT accession number P77510); 2 – *E. coli* turgor-sensing histidine kinase KdpD (P21865); 3 – *E. coli* chemotaxis histidine kinase CheA (P07363); 4 – *E. coli* serine chemotaxis receptor Tsr (P02942); 5 – *E. coli* redox chemotaxis receptor Aer (P50466); 6 – *E. coli* PTS glucose-specific EIICB component (PtsG, P69786); 7 – *Anabaena variabilis* serine/threonine protein kinase with CHASE2 sensor domain (Q3M3S5); 8 – *Leptospira interrogans* sigma regulation protein RsbU (Q8F087); 9 – *Pseudomonas aeruginosa* adenylate cyclase CyaB (PA3217, Q9HZ23); 10 – *E. coli* diguanylate cyclase AdrA (YaiC, P0AAP1); 11 – *E. coli* c-di-GMP phosphodiesterase Dos (P76129); 12 – *E. coli* c-di-GMP phosphodiesterase YcgF (P75990). Domain names are as in **Tables 3** and **4**; striped boxes indicate transmembrane segments; dotted shapes indicate integral membrane domains. Pfam entries for other domains are as follows: USP, PF00582; CheW, PF01584; TarH, PF02203. Domain sizes are not necessarily drawn to scale.

Types of Bacterial Receptors and Signaling Pathways

Public Resources on Signal Transduction

The current knowledge of signal transduction comes from a combination of genetic and biochemical studies, primarily on such model organisms as *E. coli*, *Bacillus subtilis*, *Caulobacter crescentus*, *Myxococcus xanthus*, *Pseudomonas aeruginosa*, *Synechocystis* sp., and *Halobacterium halobium*, sequence analysis of signal transduction proteins, and from structural characterization of at least some of these proteins. Comparative analysis of protein sequences played a key role in the original identification of the receiver (phosphoacceptor) domain in several different response regulators, which paved the way to the discovery of two-component signal transduction. In the past several years, availability of complete genome sequences led to a dramatic expansion of the list of potential organisms for studying signal transduction. Even more importantly, analysis of genome sequences allowed a careful accounting of all signal transduction proteins encoded in any given genome. This analysis revealed previously uncharacterized receptor proteins even in such favorite model organisms as *E. coli* and *B. subtilis*. It also showed the complexity of signal transduction systems encoded in these two organisms to be relatively modest, compared with the signaling repertoire of *P. aeruginosa* and *Synechocystis* sp., not to mention the enormous expansion of signaling genes in the genome of *M. xanthus*.

The diversity and complexity of the bacterial signal transduction machinery make systematic description of its components in any given organism a very difficult task (for *E. coli*, such a description is provided at the end of this article). This has led to the creation of public databases that attempt to collect information on the organization of signal transduction systems in various organisms, particularly those with completely sequenced genomes. Several such databases are freely available online (**Table 1**).

The first signaling database, The Histidine Protein Kinases Reference Page, was created by Thorsten Grebe and Jeffry Stock at Princeton University as supplementary material to their review of histidine kinases, published in 1999 in *Advances in Microbial Physiology*. This database, the first comprehensive classification of histidine kinases and their cognate response regulators, is still available at the web site of the University of Kaiserslautern in Germany. Although this listing has not been updated since 2000, its definition of 11 principal families of histidine kinases remains a useful tool in their analysis. The Microbial Signal Transduction (MiST) database, maintained at the Oak Ridge National Laboratory in Oak Ridge, Tennessee, contains information on the signal transduction proteins from all completely sequenced prokaryotic genomes, identified by their domain architecture. The Sensory Signal Transduction Proteins (Sentra) database, maintained at the Argonne National Laboratory in Argonne, Illinois, also lists predicted signal transduction proteins from all completely sequenced prokaryotic genomes. It also includes a separate section with manually curated annotations of the major classes of signal transduction proteins. Kyoto Encyclopedia of Genes and Genomes (KEGG), maintained by the Bioinformatics Center of Kyoto University in Japan, is a comprehensive genomic database on all completely sequenced genomes, prokaryotic and eukaryotic. One of its sections provides a graphical representation of all bacterial two-component systems. Kinases in Genomes (KinG) database, maintained at the Indian Institute of Science in Bangalore, India, provides a comprehensive listing of Ser/Thr/Tyr kinases and related proteins encoded in various prokaryotic and eukaryotic genomes. Finally, Signaling Protein Census and Response

Table 1 Electronic resources on bacterial signal transduction

Name, URL	*Comment*[a]
Specialized databases of signal transduction proteins	
Microbial Signal Transduction (MiST), http://genomics.ornl.gov/mist/	Predicted signal transduction proteins from all completely sequenced prokaryotic genomes
Sensory Signal Transduction Proteins (Sentra), http://compbio.mcs.anl.gov/sentra/	Predicted signal transduction proteins from all completely sequenced prokaryotic genomes
Kyoto Encyclopedia of Genes and Genomes (KEGG), http://www.genome.ad.jp/kegg/pathway/ko/ko02020.html	Graphical representation of two-component systems encoded in all completely sequenced prokaryotic genomes
The Histidine Protein Kinases Reference Page, http://www.uni-kl.de/FB-Biologie/AG-Hakenbeck/TGrebe/HPK/HPK.html	Classification of histidine kinases and their cognate response regulators. Last updated in 2000
Kinases in Genomes (KinG), http://hodgkin.mbu.iisc.ernet.in/~king	Ser/Thr/Tyr kinases encoded in completely sequenced genomes of prokaryotes and eukaryotes
Signaling Protein Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html	A listing of signal transduction proteins encoded in completely sequenced genomes of 330 prokaryotic species
Response Regulator Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html	A listing of response regulators encoded in completely sequenced genomes of 330 prokaryotic species
Protein family and domain databases	
Protein Families database (Pfam), http://pfam.sanger.ac.uk/	An extensive collection of protein domains, includes many signal transduction domains
Simple Modular Architecture Research Tool (SMART), http://smart.embl.de/	Protein domain database with special emphasis on prokaryotic and eukaryotic signal transduction domains
Protein Domains database (ProDom), http://prodom.prabi.fr/	Protein domain database with an exhaustive listing of automatically delineated protein domains
Conserved Domain Database (CDD), http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml	Protein domain database with curated domain alignments that are based on known 3D structures
Integrated database of protein families, domains and functional sites (InterPro), http://www.ebi.ac.uk/interpro/	An umbrella database that combines results from several different protein domain and sequence motif databases

[a] Detailed descriptions of these databases with up-to-date references are available at the respective websites.

Regulator Census, two web sites maintained by the author at the NCBI, part of the National Institutes of Health in Bethesda, Maryland, provide counts of each class of signal transduction protein and response regulator in the representative genomes from 330 prokaryotic species.

Signal Transduction Pathways

Historically, experimental studies focused on two classes of membrane-associated receptor proteins, sensory histidine kinases and methyl-accepting chemotaxis proteins (MCPs). In the past several years, analyses of microbial genomes, as well as experimental studies, revealed several additional classes of bacterial receptors, which include Ser/Thr/Tyr protein kinases and protein phosphatases, adenylate cyclases, diguanylate cyclases, and cyclic dimeric bis-(3′-5′)-guanosine monophosphate (c-di-GMP)-specific phosphodiesterases (**Table 2**).

The signaling pathways utilized by various bacterial receptors are shown in **Figure 2**. Signaling by a histidine kinase is usually referred to as two-component signal transduction, as it includes phosphoryl transfer between two different proteins, a histidine kinase and a response regulator. Two-component signal transduction pathways are extremely diverse and always include the following common steps: (1) ATP-dependent phosphorylation of a conserved His residue in the molecule of the kinase; (2) transfer of the phosphoryl residue to a conserved Asp residue in the molecule of response regulator; (3) conformational change in the response regulator that alters its interaction with the target. A majority of response regulators serve as transcriptional regulators and bind to the chromosomal DNA, but there are response regulators with alternative targets (see below).

Chemotaxis signaling, which starts from MCPs, is a special kind of two-component signal transduction that involves a specialized histidine kinase CheA, which directly interacts with MCPs, and a specialized response regulator CheY that consists of stand-alone receiver domain without any output domains. Regulation of flagellar motility is based on the interaction of the phosphorylated form of CheY with the FliM protein at the base of the flagellum, which affects the direction of flagellar rotation and thus regulates the chemotaxis response. In archaea, whose flagella are unrelated to the bacterial ones, as well as in the bacteria that move at the expense of type IV pili or utilize other non-flagellar means of transportation, such as gliding motility, the targets for CheY binding remain obscure. Recent studies revealed that signaling roles of certain MCPs go beyond chemotaxis. For example, in *M. xanthus*, an MCP has been shown to participate in regulation of developmental gene expression, whereas in *P. aeruginosa*, methyl-accepting protein WspA turned out to be part of a chemosensory system that regulates biofilm formation.

Components of the phosphoenolpyruvate (PEP)-dependent sugar:phosphotransferase system (PTS) participate in phosphorylative sugar uptake and are rarely considered to be part of the signal transduction machinery. Nevertheless, two members of the PTS phosphorelay play key roles in signal transduction. The phosphorylation level of the PTS enzyme I (EI) directly affects the chemotaxis machinery, whereas the phosphorylation level of the enzyme IIA^{Glc} ($EIIA^{Glc}$) modulates the activity of the adenylate cyclase, at least in the members of *Enterobacteriaceae.*

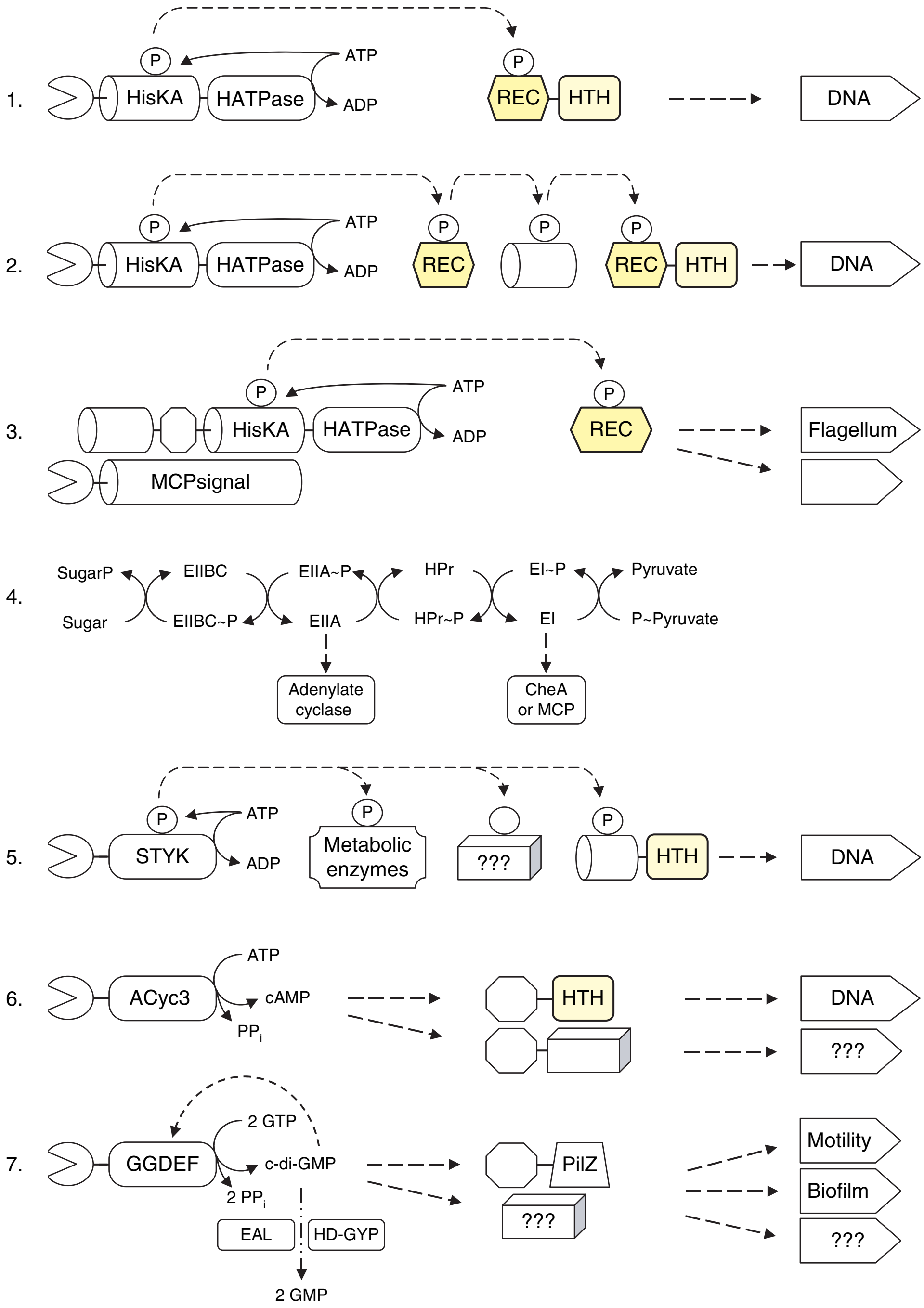
Ser/Thr protein kinases phosphorylate Ser and Thr residues in various cellular proteins. Only a small fraction

Table 2 Principal classes of bacterial receptors

Receptor type	*Function*	*Signaling mechanism*	*Phylogenetic distribution*
Histidine kinase	Regulation of transcription, other processes	Phosphorylation of the receiver domain in various proteins	Bacteria, Archaea
Methyl-accepting chemotaxis protein	Chemotaxis	Interaction with chemotaxis-specific histidine kinase CheA	Bacteria, Archaea
Ser/Thr/Tyr protein kinase	Regulation of transcription, post-translational regulation of enzyme activity, other processes	Phosphorylation of Ser, Thr, or Tyr residues in target proteins	Bacteria, Archaea
Ser/Thr/Tyr protein phosphatase	Same as above	Dephosphorylation of Ser/Thr/Tyr protein kinases or other target proteins	Bacteria, Archaea
Membrane components of the sugar: phosphotransferase system (PTS)	Sugar transport, phosphorylation, and chemotactic signaling	Direct effect on chemotaxis, most likely through direct interaction of PTS enzyme I with the histidine kinase CheA	Bacteria
Adenylate cyclase	Global regulation of transcription	Synthesis of cAMP	Bacteria
Diguanylate cyclase	Regulation of protein and polysaccharide secretion	Synthesis of c-di-GMP	Bacteria
c-di-GMP-specific phosphodiesterase	Same as above	Hydrolysis of c-di-GMP	Bacteria

of their targets have been identified so far. These include transcriptional regulators (such as actinobacterial AfsR) and metabolic enzymes (e.g., phosphofructokinase). Ser/Thr protein phosphatases reverse the effect of Ser/Thr protein kinases by dephosphorylating their target proteins or, in some cases, the Ser/Thr protein kinases themselves. Most of these enzymes are localized in cytoplasm and only a few are membrane-bound (**Table 2**).

Adenylate cyclases modulate the cellular level of cyclic adenosine monophosphate (cAMP), a key cellular second messenger (**Figure 3**), which participates in at least two different signaling pathways. One of them includes binding of cAMP to a specialized adaptor protein, CRP or CAP, triggering a conformational change in CRP that increases its affinity to DNA and allows it to activate transcription of otherwise poorly expressed genes (operons). This mechanism is sometimes referred to as catabolite repression, although in reality catabolite repression (or glucose effect) is a sum of several independently acting mechanisms. The second pathway of cAMP-mediated signaling includes its


1.
P
HisKA
HATPase
ATP
ADP
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REC
HTH
DNA
2.
P
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REC
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3.
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HisKA
HATPase
ATP
ADP
MCPsignal
P
REC
Flagellum
4.
SugarP
Sugar
EIIBC
EIIBC~P
EIIA~P
EIIA
HPr
HPr~P
EI~P
EI
Pyruvate
P~Pyruvate
Adenylate cyclase
CheA or MCP
5.
P
STYK
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ADP
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Metabolic enzymes
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HTH
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6.
ACyc3
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cAMP
PP_i
HTH
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GGDEF
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EAL
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PilZ
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Motility
Biofilm
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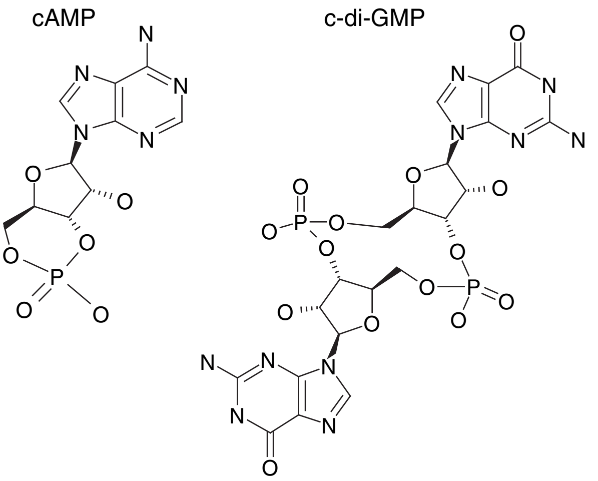

Figure 3 Structures of cAMP and c-di-GMP.

binding to specialized cAMP-binding domains on various proteins, including certain transcriptional regulators. The mechanisms of this signaling still remain largely obscure but probably involve changes in gene expression and/or protein–protein interactions in response to cAMP binding. There are several different classes of adenylate cyclases, of which only class III enzyme has been shown to function as a transmembrane receptor.

Signaling through diguanylate cyclases includes modulation of the cellular level of another cellular second messenger, c-di-GMP, which regulates a variety of functions related to the cell surface elements, including motility, secretion of proteins and exopolysaccharides, biofilm formation, and production of certain virulence factors. Some of the c-di-GMP functions are mediated by its binding to the recently described PilZ domain, while others might involve other binding proteins, including diguanylate cyclases themselves. C-di-GMP-specific phosphodiesterases, which catalyze c-di-GMP hydrolysis, could also function as c-di-GMP-binding proteins.

It is important to note that these signaling pathways are not entirely separate. In many organisms, there exists a cross-talk between components of different signaling pathways. For example, a significant fraction of response regulators have adenylate or diguanylate cyclases or c-di-GMP-specific phosphodiesterases as their output domains, which allows histidine kinase-mediated pathways to control the cAMP or c-di-GMP levels.

Structural Organization of Signal Transduction Proteins

A key aspect of the bacterial signal transduction machinery is its modular structure. Many signal transduction proteins consist of several individual protein domains that can be shuffled almost like the Lego blocks, resulting in a tremendous diversity of proteins.

Sensory Domains

Sensory (signal input) domains of bacterial receptors are extremely diverse, reflecting the diversity of the signals they perceive. However, despite this diversity, sequence similarity between sensory domains of different receptors

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Figure 2 Signal transduction pathways of the principal classes of bacterial receptors. 1 – Transcriptional regulation by two-component signal transduction systems usually includes a sensor histidine kinase and response regulator that consists of the phosphoryl-accepting receiver (REC) domain and a DNA-binding helix-turn-helix (HTH) domain. 2 – Signal transduction in the *Bacillus subtilis* sporulation machinery includes a histidine kinase (KinA, KinB, KinC, KinD, or KinE), response regulator (Spo0F) with a stand-alone REC domain, intermediate phosphorelay component (Spo0B), and transcriptional response regulator Spo0A that consists of REC and Spo0A-specific HTH domains. 3 – Signal transduction in the chemotactic machinery starts from a sensor (methyl-accepting chemotaxis protein, MCP) that interacts with the histidine kinase CheA, which phosphorylates the chemotaxis response regulator (CheY) with a stand-alone REC domain. Phosphorylated CheY interacts with the FliM protein at the base of the flagellum and with still unknown targets in other systems. 4 – In *Escherichia coli*, signal transduction in the phosphoenolpyruvate-dependent sugar:phosphotransferase system (PTS) involves interaction of the non-phosphorylated form of EIIA with the adenylate cyclase and of non-phosphorylated EI with the histidine kinase CheA. In *B. subtilis*, there is no adenylate cyclase and the non-phosphorylated form of EI interacts with one of the MCPs. 5 – Signal transduction from Ser/Thr protein kinases involves direct or indirect phosphorylation of various (mostly unknown) targets. Known targets of Ser/Thr protein phosphorylation include metabolic (e.g., glycolytic) enzymes and transcriptional regulators. 6 – Signal transduction from sensor adenylate cyclases involves interaction of the cAMP with specific cNMP-binding domains, found in the transcriptional regulator CRP and in some other proteins. The cAMP–CRP complex binds to the chromosomal DNA and activates transcription from a variety of weak bacterial promoters. Other targets of cAMP action remain mostly unknown. 7 – Signal transduction from sensor diguanylate cyclases involves interaction of c-di-GMP with various proteins that contain the c-di-GMP-binding PilZ domain or, possibly, alternative c-di-GMP-binding domains. The cellular level of c-di-GMP is controlled by c-di-GMP-specific phosphodiesterases, which are found in two different forms, the EAL and the HD-GYP domains, and, in some cases, as output domains of environmental sensors. In addition, c-di-GMP serves as an allosteric regulator of its own production. Targets for c-di-GMP (c-di-GMP-PilZ) action remain unknown at this time. Domain names are as in **Tables 3** and **4**. Domain architectures are simplified for clarity and domain sizes are not drawn to scale.

from different organisms allows grouping them into separate domain families. Members of the same domain family typically recognize the same – or closely related – ligands. Therefore, functional characterization of a sensory domain in one organism can be used as a basis for functional annotation of all proteins that contain the same domain. Some sensory domains have narrow substrate specificity, for example, the citrate-binding domain found in the citrate-sensing histidine kinase CitA (DpiB) of *E. coli*. Other sensory domains, such as the periplasmic domain of the dicarboxylate sensor DcuS, which is very similar to CitA, bind a number of related molecules (in the case of *E. coli* DcuS, C4-dicarboxylates and citrate). For many other domains, the range of the potential ligands – and hence the nature of the sensed signal(s) – remains unknown and their participation in environmental sensing is deduced primarily from their location as the N-terminal periplasmic domains of various transmembrane receptors.

While most characterized sensory domains are periplasmic (or extracytoplasmic), analyses of genome sequences identified a number of cytoplasmically located and membrane-embedded sensory domains (**Table 3**). Many sensory domains are found in more than one type of receptors (see below), which is how they have been identified as sensory domains in the first place. Indeed, if the same periplasmic or membrane-embedded domain is found in two different classes of receptors, for example, in histidine kinases and diguanylate cyclases, there is little doubt that this protein domain is involved in signal transduction, most likely as a sensory domain.

Unfortunately, identification of sensory domains through comparative sequence analysis rarely gives insights into the functions of these domains. Even when this does happen, the predicted function must be considered tentative until it has been verified by experimental studies. For example, *Vibrio cholerae* protein VC0303 belongs to a large family of histidine kinases that contain membrane-embedded N-terminal domains, which are very similar to Na^+-dependent permeases of proline (PutP) and pantothenate. This observation has led to a suggestion that these proteins might serve as sensors of the transmembrane gradient of Na^+ ions. This suggestion has not been experimentally tested so far, and members of this histidine kinase family are annotated in the protein databases as either sensor histidine kinases or Na^+/proline symporters.

Although the great majority of signal transduction pathways respond to environmental changes and employ periplasmic (or extracellular) sensors, a substantial fraction of bacterial receptors are strictly cytoplasmic

Table 3 Examples of bacterial sensory domains

Domain name	*Length (aa)*	*Ligand specificity*	*Protein structure*[a]	*Pfam entry*[b]
Periplasmic (extracellular)				
SBP_bac_3	∼220	Amino acids	1lag	PF00497
CitAP/DcuSpd	∼200	Citrate, C4-dicarboxylates	1p0z	Pfam-B_1145
NIT	∼250	Nitrate, nitrite	n/a	PF08376
PASTA	∼55	β-Lactams	1pyy	PF03793
PhoQ_sensor	∼180	Ca^{2+}	1yax	PF08918
Cache	∼80	Small ligands	n/a	PF02743
CHASE	∼220	Unknown	n/a	PF03924
CHASE2	∼240	Unknown	n/a	PF05226
CHASE3	∼130	Unknown	n/a	PF05227
CHASE4	∼250	Unknown	n/a	PF05228
Membrane-embedded				
5TMR-LYT	∼90	Murein derivatives (?)[c]	5TM	PF07694
7TMR-DISM	∼200	Unknown	7TM	PF07695
MHYT	∼200	Metal (?)[c]	6TM	PF03707
MASE1	∼280	Unknown	11TM	PF05231
MASE2	∼170	Unknown	5TM	PF05230
MASE3	∼160	Unknown	7TM	Pfam-B_23487
Cytoplasmic				
PAS	∼100	Heme, flavin, others	1wa9	PF00989
GAF	∼150	cGMP, others	1f5m	PF01590
BLUF	∼90	FAD	1x0p	PF04940
Globin sensor	∼150	Heme (O_2, CO, NO)	1or4	PF00042
HNOB	∼170	Heme (NO)	1xbn	PF07700
Phytochrome	∼180	Tetrapyrrole	1u4h	PF00360

[a] Protein DataBank (PDB, http://www.rcsb.org/pdb/ entry, if available, or the number of transmembrane segments; n/a, not available.

[b] Pfam database (http://www.sanger.ac.uk/Software/Pfam/) provides short descriptions of these domains and links to the underlying literature. As an example, the entry page for the domain PF00497 can be found at http://pfam.sanger.ac.uk/family?acc=PF00497. Domains in PfamB lack descriptions and have the URLs of http://pfam.sanger.ac.uk/pfamb?id=Pfam-B_1145 type.

[c] The question mark signifies unverified prediction.

enzymes that have no transmembrane segments. Enzymatic activities of such receptors, which include the *E. coli* nitrate sensor NtrB, *B. subtilis* sporulation regulators KinA, KinB, and KinC, and many others, are apparently modulated by intracellular, rather than extracellular, signals. Indeed, many of them contain N-terminal cytoplasmic sensor domains, such as PAS, GAF, and the globin-coupled sensor domain. The first two of these domains, PAS and GAF, have similar structures, characterized by a presence of a ligand-binding pocket that can accommodate a variety of small-molecule ligands, including heme, flavin (PAS), cGMP (GAF), and many others. For the globin-coupled sensor domain, heme is the only known ligand. Heme-containing PAS and globin domains can be sensors of oxygen, carbon monoxide, and NO molecules. In each case, binding of oxygen, CO, or NO to the heme molecule causes a shift in the positions of axial ligands, which triggers a significant conformational change in the domain structure. This conformational change affects interaction of the PAS (or globin sensor) domain with the downstream domains. Thus, the signal generated by oxygen, CO, or NO binding, is transmitted to the next domain and, eventually, to the C-terminally located signal transduction domains. This allows PAS-containing receptors to serve as sensors of the general energy state of the cell (e.g., *B. subtilis* sporulation regulating histidine kinase KinA), its redox state (*E. coli* aerotaxis receptor Aer) or oxygen level (*E. coli* c-di-GMP phosphodiesterase Dos). Many receptors contain more than one sensor domain (e.g., *B. subtilis* KinA contains three PAS domains), whose mode of action and hierarchy, if any, are usually unknown.

It must be noted that not every N-terminal periplasmic or integral membrane domain of a bacterial receptor molecule is necessarily involved in signaling. For example, the histidine kinase UhpB, which regulates transport and metabolism of glucose-6-phosphate and related sugars, has a membrane-embedded N-terminal domain that does not bind extracellular glucose-6-phosphate and cannot serve as its sensor. Instead, this domain interacts with another membrane protein, UhpC, which is closely related to sugar phosphate transport proteins but actually works as the true sensor of glucose-6-phosphate in the UhpB–UhpC complex. In the *E. coli* histidine kinase KdpD, which regulates membrane transport of K^+ ions, the function of the membrane segment appears to be limited to anchoring the enzyme molecule in the membrane and ensuring proper interaction between its cytoplasmically located domains, the N-terminal turgor-sensing domain and the C-terminal histidine kinase domain. Thus, for many putative sensory domains, the current functional prediction, if any, should be considered at best an educated guess. The nature of the signal(s) that they are sensing remains obscure and needs to be explored in future experimental studies.

Structural Organization of Two-Component Signal Transduction Systems

Histidine kinases

Histidine kinases are the best studied, most numerous, and most diverse membrane receptors, which control the greatest variety of cellular responses. Most of the diversity of histidine kinases comes from the sensory (signal input) domains, which can be periplasmic, membrane-embedded, or cytoplasmic. A single histidine kinase can contain several sensory domains, for example a periplasmic ligand-binding domain and one or more PAS domains in the cytoplasm (**Figure 2**). In contrast, cytoplasmic signal transduction modules of histidine kinases are rather uniform. Analysis of sequence similarities between different histidine kinases by Parkinson and Kofoid in 1992 revealed five conserved sequence motifs, referred to as H, N, G1, F, and G2 boxes. The first of these boxes corresponded to the sequence motif around the conserved phosphoryl-accepting histidine residue. These motifs are still widely used in descriptions of newly characterized histidine kinase. However, the diagnostic value of these motifs was partly undermined by discovery of proteins that lack one or more of these 'boxes' but still function as histidine kinases, such as *P. aeruginosa* AlgZ/FimS, a sensor protein controlling alginate biosynthesis regulator AlgR, or *Clostridium perfringens* VirS, a sensor protein for the virulence regulator VirR. Determination of the crystal structures of several histidine kinases revealed that they all contain two separate protein domains (sometimes there are one or more additional domains). One of them is an ATPase domain (referred to HATPase in Pfam database, see **Table 4**) that binds ATP and transfers its γ-phosphate onto a histidine residue in the other domain of histidine kinases, which is referred to as the dimerization/phosphorylation domain (His_Kinase_A or HisKA in Pfam, **Table 4**). There are several variants of HisKA domains, which, despite having similar structures, consisting of long α-helices, share little sequence similarity. Pfam database currently assigns the HisKA domain the status of a domain group, or a clan, and divides it into four separate domain families, HisKA (PF00512), HisKA_2 (PF07568), HisKA_3 (PF07730), and HWE_HK (PF07536). The HATPase domain is always located C-terminally of the HisKA domain. Accordingly, the H box of Parkinson and Kofoid maps to the HisKA domain, whereas N, G1, F, and G2 boxes map to the HATPase domain.

In addition to the sensor, HisKA, and HATPase domains, certain histidine kinases contain other domains. Periplasmic (extracytoplasmic) sensor domains are connected to the intracellular signal transduction modules by one or more transmembrane segments and, sometimes, the cytoplasmic helical linker (HAMP) domain (**Table 4**). In addition, cytoplasmic signal transduction modules of

Table 4 Properties of the signal transduction domains

Receptor type, domain name	*Length, aa*	*Protein structure*[a]	*Pfam entry*	*Function, comments*
Histidine kinase				
HAMP	∼50	2asw	PF00672	Dimerization, signal transfer. Upon dimerization, forms a four-helix bundle
HATPase	∼120	1bxd	PF02518	ATP-dependent phosphorylation of the HisKA domain. May have a phosphatase activity toward CheY-P
HisKA	∼120	1joy	PF06580 (PF00512, PF07568, PF07730, PF07536)	Phosphoacceptor domain, no enzymatic activity. Upon dimerization, forms a four-helix bundle. Can be found in several distinct variants (HisKA, HisKA_2, HisKA_3, and HWE_HK in Pfam)
HPt	∼100	1tqg	PF01627	Phosphoacceptor domain, no enzymatic activity. Upon dimerization, forms a four-helix bundle
REC (CheY)	∼120	2che	PF00072	Phosphoacceptor domain, dephosphorylates HisKA domains
Methyl-accepting chemotaxis protein (MCP)				
MCP signal	∼350	1qu7	PF00015	Dimerization domain, no enzymatic activity. Upon dimerization, forms a four-helix bundle. Can be reversibly methylated on Glu residues
Phosphotransferase system (PTS)				
Enzyme I (EI)	∼570	2hwg	PF05524, PF00391, PF02896	Phosphoenolpyruvate-dependent autophosphorylation, phosphoryl transfer to the HPr protein
HPr protein	∼90	1ptf	PF00381	Phosphoacceptor domain, no enzymatic activity
Enzyme IIA (EIIA)	∼170	1gld	PF000358	Phosphoryl transfer from the HPr protein to the EIIB components
Enzyme IIB (EIIB)	∼80	1o2f	PF000367	Phosphoryl transfer from the HPr protein (or from EIIA) to the sugar substrates
Ser/Thr protein kinase				
STYK	∼250	1jwh	PF00069	Phosphorylation of Ser and Thr residues in various target proteins. The first step is an ATP-dependent autophosphorylation
Ser/Thr protein phosphatase				
PP2C	∼250	1a6q	PF07228	Dehosphorylation of Ser and Thr residues in STYK domains and other target proteins
Adenylate cyclase				
ACyc3	∼190	1cul	PF00211	Synthesis of cAMP
cNMPbind	∼90	1hw5	PF00027	Binding of cAMP, interaction with various targets
CRP_HTH		1hw5	PF00325	Interaction with the target sites on the DNA
Diguanylate cyclase				
GGDEF	∼180	1w25	PF01590	Synthesis of c-di-GMP, inactivated domains serve as allosteric regulators
PilZ		1ywu	PF07238	Binding of c-di-GMP, interaction with various targets
c-di-GMP-specific phosphodiesterase				
EAL	∼250	2bas	PF00563	Hydrolysis of c-di-GMP, inactivated domains serve as allosteric regulators
HD-GYP	∼170	1yoy[b]	PF01966[b]	Hydrolysis of c-di-GMP

[a] Protein DataBank entry.

[b] PDB and Pfam entries for the HD-GYP domain cover only the catalytic HD subdomain.

some histidine kinases, referred to as hybrid histidine kinases, contain an additional C-terminal domain, a CheY-like phosphoacceptor (receiver, REC) domain, with a conserved aspartate residue, which is part of the signal transduction phosphorelay. Another phosphoacceptor protein domain, histidine phosphotransfer

(HPt) domain, has a four-helix-bundle structure, similar to that of HisKA, and also contains a conserved histidine residue. The HPt domain can be found at the N-terminus of the protein (e.g., in the chemotaxis histidine kinase CheA), in the middle of it, or, most often, at its very C-terminus, see **Figure 2** for examples.

Response regulators

Response regulators of the two-component signal transduction systems are diverse proteins that share the common phosphoacceptor REC domain, which is often referred to as CheY or CheY-like domain, after its best-known representative. This domain is enzymatically active: it catalyzes phosphotransfer from the histidine residues of the HisKA domains to its own conserved aspartate residues, as well as its own dephosphorylation. The combination of these two activities in the REC domains of each particular response regulator determines the half-life of the phosphorylated form of the domain (CheY∼P or, more generally, REC∼P) and hence the fraction of the response regulator molecules that are in the active (phosphorylated) conformation at any given time.

Although a great majority of response regulators combine the REC domain with some kind of a signal output domain, approximately one-sixth of all response regulators consist of a stand-alone REC domain. This group includes two well-studied proteins, the chemotaxis response regulator CheY of *E. coli* and *Salmonella enterica* and the sporulation regulator Spo0F of *B. subtilis*. Despite obvious sequence and structural similarities and participation in similar phosphorylation–dephosphorylation cycles, these two proteins have dramatically different modes of action. Chemotactic signal transduction through the first of them, CheY, relies solely on protein–protein interactions. After CheY gets the phosphoryl group from the chemotaxis histidine kinase CheA, it does not transfer this group any further. Phosphorylation shifts the CheY molecule into the active conformation that has an increased affinity to its target molecule FliM in the flagellar basal body. Non-phosphorylated CheY is also capable of interacting with FliM, albeit not as strongly. Thus, phosphorylation of CheY merely shifts the equilibrium of its two forms (there appear to be intermediate forms as well), leading to a change in the rotation pattern of the flagellum, which is reflected in altered motility pattern of the whole cell. In contrast, Spo0F is an active part of the signaling phosphorelay. It accepts the phosphoryl group from the sporulation histidine kinase KinA and, in turn, serves as a phosphoryl donor for Spo0B, which further transfers it to the sporulation response regulator Spo0A. Phosphorylation experiments *in vitro* showed that Spo0F can also donate the phosphoryl group back to the histidine kinase KinA; whether it takes place *in vivo* remains unknown. Given the existence of phosphatases, specifically dephosphorylating Spo0F∼P, Spo0F appears to serve as a phosphate sink, a checkpoint in the control of sporulation process. According to the Response Regulator Census (see **Table 1**), many bacterial genomes encode multiple response regulators with the stand-alone REC domains (e.g., there are 42 such genes in *M. xanthus* and 38 in *Solibacter usitatus*). It is likely that some of them, like CheY, transmit the signal through protein–protein interactions, whereas others, like Spo0F, participate in complex signaling phosphorelays.

With the exception of members of the CheY/Spo0F protein family, all other response regulators are two-domain (or even three-domain) proteins that combine the REC domain with a signal output domain, which is usually located at the C-terminus of the polypeptide chain. Most of these proteins are transcriptional regulators that activate or repress transcription of specific target genes. Accordingly, the most common output domains bind DNA or RNA, although some response regulators have enzymatic or ligand-binding output domains.

The most common DNA-binding response regulators belong to the OmpR/PhoB family and have a winged helix-turn-helix (wHTH) DNA-binding domain (**Table 5**). The second in abundance is the NarL/FixJ family of response regulators, which have a typical helix-turn-helix (HTH) DNA-binding output domain. Less common DNA-binding response regulators contain DNA-binding output domains of the Fis type (NtrC and ActR/PrrA families), AraC type (YesN family), LytTR type (LytR/AgrA family), Spo0A_C type (Spo0A family), and several others. There are families of response regulators that are found just in one or two instances in the current protein databases. In each case, phosphorylation of the REC domain favors its transition into an active conformation and/or its dimerization. Dimerization of response regulators is a key mechanism of the transcriptional regulation by two-component systems, as response regulator dimers have a higher affinity to the tandem (or palindromic) transcriptional regulator binding sites on the chromosome. Within each family of response regulators, the signaling specificity is determined by the tight interaction of the REC domains with their cognate histidine kinases and of the HTH domains with the target sites on the DNA. As a result, transcriptional regulators with dramatically different biological functions (e.g., OmpR and PhoB) can have very similar sequences. This is why sequence-based assignments of biological function are often unreliable, despite confident recognition of the DNA-binding output domains in most response regulators.

In a significant fraction of response regulators, the output domains are enzymatic, such as the methylesterase domain in the chemotaxis response regulators of the CheB family. These output domains are typically the same as in bacterial environmental receptors: adenylate and diguanylate cyclases, c-di-GMP-specific phosphodiesterases, PPC2-type Ser/Thr protein phosphatases, and even histidine kinases. These types of domains are discussed in detail in the following sections. It is

Table 5 Signal output domains in bacterial response regulators[a]

Response regulator family	*Output domain*	*Length, aa*	*Function*	*Protein structure*[b]	*Pfam entry*	*% Total*[c]
Stand-alone						
CheY/Spo0F	None	$\sim$120	Protein–protein interaction, phosphorelay	2che	PF00072	16.8%
DNA-binding						
OmpR/PhoB	wHTH	$\sim$120	Transcriptional regulation	1bl0	PF00486	29.6%
NarL/FixJ	HTH	$\sim$120	Transcriptional regulation	1rnl	PF00196	16.5%
LytR/AgrA	LytTR	$\sim$110	Transcriptional regulation	3bs1	PF04397	2.9%
ActR/PrrA	Fis (HTH_8)	$\sim$50	Transcriptional regulation	3fis	PF02954	1.1%
AraC/YesN	HTH_AraC	$\sim$140	Transcriptional regulation	1bl0	PF00165	0.7%
PhyR	RpoE	$\sim$140	Transcriptional regulation	1or7	PB13556 PF08281	0.4%
Spo0A	Spo0A_C	$\sim$120	Transcriptional regulation	1fc3	PF08769	0.2%
GlnL	YcbB	$\sim$140	Transcriptional regulation	n/a	PF08664	0.2%
Three-domain DNA-binding						
NtrC	AAA, Fis (HTH_8)	$\sim$230 $\sim$50	Transcriptional regulation	1ojl, 3fis	PF00158 PF02954	9.7%
SARP	HTH, BTAD	$\sim$220	Transcriptional regulation	2fez	PF00486 PF03704	<0.1%
RNA-binding						
AmiR/NasR	ANTAR	$\sim$55	Regulation of transcription (anti)termination	1qo0	PF03861	1.0%
RB8820	CsrA	$\sim$60	Regulation of translation and mRNA decay	1vpz	PF02599	<0.1%
Enzymatic						
CheB	CheB	$\sim$180	Methylesterase	1chd	PF01339	2.6%
FliY	CheC	$\sim$200	Asp$\sim$P phosphatase	1squ	PF04509	0.2%
PA0267	HDOD		Predicted phosphodiesterase	1vqr	PF08668	0.2%
Nfa52850	TrxB	$\sim$220	NAD(P)H:disulfide oxidoreductase	1tde	PF00070	0.1%
Protein-binding						
CheV	CheW	$\sim$140	Protein–protein interaction	1k0s	PF01584	1.4%
PatA	PATAN	$\sim$160	Protein–protein interaction	n/a	n/a	0.2%

[a] This listing does not include GGDEF, EAL, HD-GYP, AC3, or PP2C output domains, listed in **Table 4**.

[b] Protein DataBank entry, if available; n/a, not available.

[c] Fraction of the response regulators from this family among 9083 response regulators encoded in complete genomes of 330 prokaryotic species, see Response Regulator Census, http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html.

important to note, however, that such response regulators put other signaling mechanisms under the control of the two-component signal transduction. Indeed, a response regulator with an adenylate cyclase output domain could control the cellular cAMP level irrespective of the status of receptor adenylate cyclases. This could be the case also for the control of c-di-GMP level. The case of Ser/Thr protein phosphorylation is even more illuminating. Response regulators with the PPC2-type Ser/Thr protein phosphatase domain far outnumber those with a protein kinase domain. The two-component signaling system apparently controls the level of protein phosphorylation on serine and threonine residues by regulating the rate of their dephosphorylation.

Some response regulators consist of more than two domains. In transcriptional regulators of the NtrC family, the N-terminal REC domain and the C-terminal DNA-binding Fis-like domain are separated by the central AAA-type ATP-binding domain, whose ATPase activity is required for the DNA-binding. The response regulator VieA from *V. cholerae* contains two output domains, a c-di-GMP-specific phosphodiesterase (EAL) and a DNA-binding HTH domain. In that case, the role of the HTH domain, if any, remains to be established.

In summary, bacterial response regulators contain a wide variety of output domains that put the histidine kinases at the top of signaling hierarchy, allowing the cell to control its metabolism and behavior in response to various environmental challenges.

Alternative Signal Transduction Systems

Methyl-accepting chemotaxis proteins

MCPs contain a characteristic signal transduction domain (Pfam domain PF00015) that consists of two very long antiparallel α-helices, connected by a U-turn. The

U-turn serves as a signaling module and contains a characteristic highly conserved sequence motif IAxQTNLLALNAAIEAARAGExGRGFAVVAxEVRx-LA. It directly interacts with the chemotaxis histidine kinase CheA, modulating its activity. Thus, in the MCP–CheA assembly, the whole MCP can be considered a sensor domain of CheA. Despite giving the MCP its name, methylation does not seem to be a necessary part of the signal transduction mechanism. Rather, methylation and demethylation change the charge distribution along the α-helices and affect the packing of these α-helices against each other, which, in turn, is reflected in the interaction between MCP and CheA. This adds yet another regulatory circuit to the chemotaxis signaling machinery.

Phosphotransferase system components

The PTS catalyzes uptake of certain sugars, coupling membrane transport of its substrates with their phosphorylation. In addition to its transport function, the PTS is an important component of the signaling machinery that controls chemotaxis to its sugar substrates. Like histidine kinases, PTS proteins are phosphorylated on the histidine residue. However, in contrast to the ATP-His-Asp or ATP-His-Asp-His-Asp phosphorelay, typical for the two-component signaling, the PTS phosphorelay starts from PEP and includes only His residues (at least, in EI, HPr, and EIIA components). The high free energy of PEP hydrolysis ensures that in the absence of carbohydrate substrates all PTS components stay in the phosphorylated form. The limiting step in the whole phosphorelay appears to be PEP-dependent autophosphorylation of the first component, EI. Therefore, in the presence of carbohydrate substrates, phosphoryl flow through the PTS components occurs at a higher rate than re-phosphorylation of EI by PEP. As a result, EI, HPr, and EIIA components become partly dephosphorylated, which serves as a signal both for the chemotaxis machinery and for the *E. coli* adenylate cyclase. Although any direct interaction between PTS components and MCP or CheA remains to be demonstrated, the available data suggest that unphosphorylated EI can interact with one or more MCPs (in *B. subtilis*) or with CheA (in *E. coli*), modulating the CheA activity and, hence, the cellular level of CheY$\sim$P. The second mechanism of signal transduction from the PTS involves EIIA$^{\mathrm{Glc}}$. This protein has been shown to interact with the adenylate cyclase and other targets, including the lactose permease. In some organisms, PTS-mediated signaling includes additional control mechanisms, such as reversible phosphorylation of the HPr protein on its Ser residue, which affects the ability of this protein to participate in the PTS phosphorelay.

Ser/Thr/Tyr protein kinases and protein phosphatases

Reversible protein phosphorylation on serine, threonine, or tyrosine residues is a key regulatory mechanism in eukaryotic cells. In the past several years, Ser/Thr protein kinases have been recognized in a variety of prokaryotic cells but are still often referred to as 'eukaryotic-type' protein kinases. In fact, Ser/Thr protein kinases appear to be the only (known) type of receptors encoded in many archaeal genomes and the principal type of signal transduction proteins in such bacteria as *Frankia alni* or *Rhodopirellula baltica*. The active domain of Ser/Thr/Tyr kinases (Pfam domain PF00069) is a member of a vast superfamily that also includes aminoglycoside phosphotransferase, choline kinase, lipopolysaccharide kinase, and 3-deoxy-D-*manno*-octulosonic acid (KDO) kinase domains. Nevertheless, Ser/Thr kinases are relatively easy to recognize, particularly in such genomes as *M. xanthus*, which encodes more than 100 of them, almost as many as histidine kinases. Despite their abundance, Ser/Thr protein kinases are still poorly studied; only a handful of their targets have been recognized and the importance of this kind of regulation often gets overlooked. A recent survey of phosphorylated proteins in *B. subtilis* suggested that most glycolytic enzymes, as well as several enzymes of the tricarboxylic acid cycle and the pentose phosphate pathway, can be phosphorylated on serine or threonine residues and, theoretically, could be subject of regulation by Ser/Thr protein kinases. Given that *B. subtilis* genome encodes only four Ser/Thr protein kinases, they might have rather wide substrate specificity.

Bacterial Ser/Thr protein phosphatases are primarily of the PP2C-type. Cellular targets for most of them remain unknown, although some of them have been shown to reverse the action of their cognate Ser/Thr protein kinases, possibly by dephosphorylating Ser/Thr kinases themselves.

Adenylate cyclases

Adenylate (adenylyl) cyclases, the enzymes that produce cAMP from ATP, exist in several unrelated variants, referred to as classes. Traditionally, the enzyme from *E. coli* is considered class I adenylate cyclase. It is a soluble enzyme that does not seem to sense any environmental signals. However, its activity is modulated by the EIIA$^{\mathrm{Glc}}$ component of the glucose-specific phosphotransferase system (PTS). The phosphorylated form of EIIA$^{\mathrm{Glc}}$ appears to activate adenylate cyclase, whereas the dephosphorylated form, accumulating in the presence of extracellular glucose, does not bind to the adenylate cyclase or even inhibits it. Thus, in the presence of glucose or other PTS sugars, adenylate cyclase activity decreases, leading to a drop in the cellular level of cAMP. This is one of the mechanisms contributing to the phenomenon of catabolite repression.

Bacterial receptor-type adenylate cyclases belong to the so-called class III and were first described in 1996 in the cyanobacterium *Spirulina platense*. Analysis of the gene sequence predicted that its product would contain a signal peptide, a periplasmic sensor domain, a membrane-spanning domain, and an adenylate cyclase-like catalytic domain. A similar gene from another cyanobacterium, *Anabaena* sp. PCC7120, was shown to complement a cya^- mutant of *E. coli*, indicating that it encoded an enzymatically active adenylate cyclase domain. Receptor-type adenylate cyclases were later found in a variety of diverse organisms, including the actinobacteria *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, α-proteobacteria *Bradyrhizobium japonicum* and *Rhizobium leguminosarum*, δ-proteobacteria *M. xanthus* and *Stigmatella aurantiaca*, and the spirochete *Treponema denticola*. In addition, certain adenylate cyclases were found to have membrane-embedded sensor domains. One of them, the *P. aeruginosa* protein CyaB (PA3217), proved to be a key regulator of the expression of virulence factors, contributing to mouse infection by *P. aeruginosa*.

Diguanylate cyclases and c-di-GMP phosphodiesterases

A recently identified group of bacterial membrane receptors includes proteins with GGDEF, EAL, and HD-GYP signal transduction domains that synthesize and hydrolyze the second messenger c-di-GMP (**Figure 3**). These proteins have been first recognized as sensor proteins through computational analysis of bacterial genomes. Subsequent experimental studies revealed their role in regulating biofilm formation, development of flagellar apparatus, and a variety of other processes.

The GGDEF domain has been identified as a diguanylate cyclase that synthesizes c-di-GMP from two molecules of GTP. This enzyme has been recently purified and crystallized, its three-dimensional structure is available in the Protein DataBank (see **Table 4**). Hydrolysis of c-di-GMP can be carried out by two different classes of c-di-GMP-specific phosphodiesterases, referred to as the EAL and HD-GYP domains. The HD-GYP domain is a member of the HD phosphohydrolase domain family (Pfam domain PF01966) and consists of a moderately conserved phosphodiesterase subdomain and a highly conserved but uncharacterized subdomain, which probably accounts for the substrate specificity of the protein. HD-GYP is recognized as a separate domain in the Conserved Domain database (CDD), but not in Pfam.

Almost a half of all bacterial GGDEF domains and two-thirds of all EAL domains are found in fused proteins that contain both GGDEF and EAL domains. The overall activity of these fusion proteins can be either synthesis or hydrolysis of c-di-GMP, as has been observed when these proteins were first characterized in 1998 by Moshe Benziman and colleagues. It appears that in many such fusion proteins, at least one of the domains is enzymatically inactive and serves as an allosteric regulator of the other one. In some cases, however, both domains appear to be active. While some of the GGDEF, EAL, and HD-GYP domains comprise the signal transduction modules of membrane-anchored environmental sensors, they are often found in cytoplasmic proteins, including many response regulators.

Interaction of Signal Transduction Pathways

As mentioned above, different classes of bacterial receptors sometimes contain similar sensory domains. This phenomenon is often observed between different groups of organisms. Thus, histidine kinases with CHASE4 sensor domain are found mostly in archaea, whereas diguanylate cyclases with CHASE4 sensor domain are found exclusively in bacteria (**Table 6**). This observation suggests that different organisms employ different signaling pathways and, ultimately, different mechanisms to respond to the same kinds of environmental signals. For example, *Thiomicrospira denitrificans* that senses extracellular nitrate by a NIT sensor domain of a histidine kinase (**Table 6**) would generate an intracellular response, transcriptional or otherwise, modulated by its cognate response regulator. *Shewanella amazonensis*, which encodes an NIT-coupled MCP, would respond to nitrate by altering its chemotactic behavior, whereas *Chromobacterium violaceum*, encoding a NIT-coupled diguanylate cyclase, is most likely to react to nitrate by adjusting its motility, protein, or polysaccharide secretion. Thus, the diversity of signal transduction pathways ensures that different organisms respond to similar or even identical environmental signals by launching a variety of cellular responses. These cellular responses become particularly complex in free-living organisms with large genomes, which encode a particularly large number of signal transduction proteins. In such organisms diverse receptors with common sensory domains can often coexist, providing a multi-level response to environmental challenge. For example, the genome of *Anabaena* sp. PCC 7120 encodes 13 proteins with the CHASE2 domain, including four histidine kinases (All1178, All3347, All5309, and Alr5151), two Ser/Thr protein kinases (All4838 and Alr1869), an adenylate cyclase *(*All1118), and a diguanylate cyclase (All1219). Although the environmental signal sensed by the CHASE2 domain has not been identified, there are certain indications that it might be osmolarity or a related parameter. It would be hardly surprising if *Anabaena* sp., a freshwater cyanobacterium with complex metabolic capabilities, would be able to elicit more than one response to the changes in the osmotic pressure around it.

Table 6 Diversity of the sensor–signal transduction domain combinations in bacterial receptors

Domain name[a]	*HisK*	*MCP signal*	*STYK*	*PP2C*	*ACyc3*	*GGDEF*	*GGDEF-EAL*	*EAL*	*HD-GYP*	*Other*
Extracellular										
SBP_bac_3	GSU2755	–	SCO4911	EAY27325	–	SO_3700	PP_0386	CPE0914	TM_1170	SACE_4896
NIT	Tmden_1926	Sama_1713	–	–	*CG5719*	CV_3252	SO_0141	–	–	Bcen_5756
Cache	BLi00440	TM1428	–	Mll6700	Lpg1322	VVA0051	VV0195	DVU_0457	PP_2599	DVU_2637
CHASE	MXAN_6941	–	*AAS78641*	*BAF29743*	*AAA33164*	VVA0456	VV0017	–	–	
CHASE2	PA4036	–	Ava_4764	–	Lpg1739	DR_1633	Bll6124	–	–	
CHASE3	DRA0205	GSU0683	–	FRAAL1878	BAA22996	GSU1937	–	–	–	
CHASE4	RL3120	–	–	–	–	PA0847	GSU2511	–	–	Gura_0265
Membrane-embedded										
5TMR-LYT	VV0692	–	–	Swol_1793	–	DR_1090	SO_1500	–	–	Jann_0472
7TMR-DISM	Gura_0744	TP0040	–	LBJ_0248	LBJ_0018	SO_1570	Bll1502	–	–	LBJ_2951
MHYT	Sden_3722	–	–	–	–	Bcen_1563	PA1727	–	–	SPO2400
MASE1	MXAN_6855	–	–	Mmcs_3900	–	Slr1798	PA1181	–	–	DVU3106
MASE2	–	–	–	–	PA3217	STM0385	–	–	–	–
Cytoplasmic										
PAS	PA5124	Aer_Ecoli	–	BSU34110	Tery_3993	Lpg0155	BCE_0696	Arth_1906	DVU0129	PtsP_Ecoli
GAF	Ava_1719	GSU1704	Arth_0192	LB112	LA2834	SO_3759	Mflv_4377	–	VV2051	ABB34328
Globin sensor	MXAN_4246	Amb2517	*CAK86513*	–	*CAJ04664*	YddV_Ecoli	CAI07755	Bxe_B2627	–	VVA0580
Phytochrome	DRA0050	–	BAE20158	Mflv_4519	–	RPB_2169	RSP_4111	–	–	XOO0264

[a] The domain names are as in **Tables 3** and **4**. Each cell contains an example of a protein with the specified domain combination, listed by its gene name or accession number in the NCBI protein database. The names in italics indicate domain combinations found so far only in eukaryotes. Dashes indicate domain combinations not found in the NCBI protein database as of 1 July 2007.

Another mechanism of cross-talk between different signaling pathways involves response regulators with enzymatic output domains. For example, environmental signal perceived by the *P. aeruginosa* methyl-accepting protein WspA is transmitted through a hybrid histidine kinase WspE to the response regulator WspR that has a diguanylate cyclase (GGDEF) output domain and regulates biofilm formation. This way, signals from such receptors as MCPs or histidine kinases may end up regulating a variety of diverse processes.

In addition, genome analysis reveals numerous cases of signaling proteins that combine different kinds of signal transduction domains, for example, an adenylate cyclase with a Ser/Thr protein kinase. Such proteins are very difficult to annotate, let alone figure out their cellular functions. Nevertheless, preservation of such multidomain proteins through millions of years of evolution indicates that they do have a function, at least in some environmental conditions. It would be reasonable to suggest that the extreme diversity of signal transduction proteins, generated by numerous combination of a relatively small number of the core protein domains, reflects different paths of adaptation to their environment, selected in different bacterial lineages.

How *E. coli* Sees the World?

Despite many years of studies, our understanding of signal transduction pathways is still very limited, even in the best-studied model organisms such as *E. coli* and *B. subtilis*. We still lack a clear understanding which environmental parameters these organisms sense, let alone why they choose these parameters and not others. However, the availability of complete genomes gives us an opportunity to list all components of the signaling network and start analyzing and modeling their respective contribution to the regulation of gene expression and cellular behavior. Several years ago, James Hoch and colleagues published a review on signal transduction in *B. subtilis* (see ‘Further Reading’) entitled ‘How one organism sees its world’. In the same fashion, it could be instructive to look at the genome of *E. coli* and see what receptors it uses to monitor the environment and the intracellular milieu. To the best of our knowledge, the signal transduction machinery of *E. coli* includes the following components:

- 30 histidine kinases with 32 response regulators
- 23 membrane components of the sugar PTS
- 12 diguanylate cyclases
- 10 c-di-GMP phosphodiesterases
- 7 proteins with both GGDEF and EAL domains
- 5 MCPs
- 2 predicted Ser/Thr protein kinases
- 1 class I adenylate cyclase

How much do we know about their functions? The signals sensed by each of the five MCPs have been experimentally characterized and are as follows:

- Tsr – serine
- Tar – aspartate, maltose
- Trg – ribose, galactose
- Tap – dipeptides
- Aer – redox state of the respiratory chain

The last of these MCPs, Aer, is obviously important for sensing the presence of usable terminal electron acceptors, reflecting the choice between a respiratory and fermentative metabolism. The ligands sensed by the other four MCPs include amino acids, peptides, and sugars. However, it is hard to say why evolution has chosen these particular ligands over other amino acids and sugars. For example, why serine and aspartate are more important for chemotaxis than glutamine and asparagine, which link carbon and nitrogen metabolism? Likewise, why maltose and galactose and not glucose or fructose? A partial answer to the second question is given by the listing of substrates of the sugar PTS, which serves as an MCP-independent mechanism of regulating chemotaxis:

- FruA, FrvB, FrwC, HrsA, YpdG – fructose
- AscF, CelB – cellobiose (β-D-Glu-D-Glu)
- MtlA, CmtA – mannitol
- GatC, SgcC – galactitol
- AgaC/D, AgaW – *N*-acetylgalactosamine
- NagE – *N*-acetylglucosamine
- ManX/Y – mannose
- SrlA – sorbitol
- PtsG – glucose
- MalX – maltose (α-D-Glu-D-Glu)
- TreB – trehalose (α-D-Glu-D-Glu)
- GlvC – α-glucosides
- BglF – β-glusosides
- SgaB – ascorbate
- YfeV – *N*-acetylmuramic acid

Thus, *E. coli* carries in its genome genes encoding chemotaxis receptors for almost any commonly found monosaccharide and several disaccharides. Whether these genes are constitutively expressed at sufficient levels to contribute to the cell behavior remains an open question. It appears that at least some of the PTS receptor genes need to be induced by the corresponding sugar. Regarding transcriptional regulation, signals for 24 out of 30 histidine kinases have been characterized. These signals (ligands) are as follows:

- CheA – chemotaxis
- BaeS, BasS, CpxA, EvgS, RcsC, RscD – envelope stress
- EnvG, KdpD – osmotic stress, K^+ gradient
- PhoM, PhoQ, PhoR – phosphate (and/or Ca^{2+}, Mg^{2+})

- NarQ, NarX – nitrite/nitrate
- CusS, ZraS – heavy metals (Cu^+/Ag^+, Zn^{2+}/Pb^{2+})
- ArcB, BarA – O_2, H_2O_2
- CitA – citrate
- DcuS – fumarate, C4-dicarboxylates
- UhpB – glucose-6-phosphate
- GlnL – glutamine
- TorS – trimethylamine oxide
- QseC – quorum sensing

The signals for the remaining six histidine kinases – AtoS, RstB, YehU, YpdA, YfhK, and YedV – remain unknown. AtoS has been shown to regulate the biosynthesis of poly-3-hydroxybutyrate upon induction with acetoacetate, whereas the other five have unknown functions. It is remarkable how many histidine kinases are sensing either envelope and osmotic stress or redox state of the cell and the availability of terminal electron acceptors. The fact that these histidine kinases coexist in the same cell suggests a certain degree of sophistication in their interactions, seen, for example, in the complex division of functions between NarQ and NarX. In most cases, however, the hierarchy between different sensors, if any, remains unknown.

Our current knowledge of the functions of 29 *E. coli* proteins with GGDEF and/or EAL domains that function as diguanylate cyclases and/or c-di-GMP-specific phosphodiesterases is far more limited. The sensed ligand, oxygen (and potentially CO and NO), has been established only for one of them, YddU, which was accordingly renamed 'direct oxygen sensor', or Dos. Several other GGDEF and/or EAL domain proteins, such as YaiC (AdrA), YdaM, YciR, and YhdA, have been shown to regulate, respectively, cellulose biosynthesis, production of curli fimbriae, and carbon storage. For other GGDEF and/or EAL domain proteins (Rtn, YcdT, YddV, YdeH, YeaI, YeaJ, YeaP, YedQ, YegE, YfeA, YfgF, YfiN, YhjK, YliF, YneF, YahA, YcgF, YcgG, YdiV, YhjH, YjcC, YlaB, YliE, and YoaD), neither the sensed signal nor the regulated process are known at this time.

The situation with the two predicted Ser/Thr protein kinases of *E. coli* is not much better. Although these proteins have been identified as members of the Ser/Thr protein kinase superfamily and have all the key active site residues intact, neither of them has been actually shown to function as a Ser/Thr protein kinase. One of them, UbiB, is required for a hydroxylation step in ubiquinone biosynthesis and was initially thought to function as 2-octaprenylphenol hydroxylase. However, this enzymatic activity has not been experimentally demonstrated. Thus, it remains unknown at this time whether UbiB is an enzyme of ubiquinone biosynthesis or a Ser/Thr protein kinase that regulates this process. The functions of the second predicted Ser/Thr protein kinase, YegI, also remain unknown.

Summing up, there remain major puzzles even in signal transduction pathways of *E. coli*. For most other bacteria free-living bacteria, understanding the signal transduction mechanisms and pathways will remain a challenge for years to come.

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Spirochetes

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Glossary

chemotaxis The movement along a chemical concentration gradient either toward or away from a chemical stimulus.
commensal An organism participating in a relationship in which that species derives benefit while the other is unaffected.
microbiome The entourage of associated microflora in a host.
parasite An organism participating in a relationship in which that species derives benefit while the other is harmed.
pathogenesis The process by which a disease occurs.
saprophyte An organism that grows on and derives its nourishment from dead or decaying organic matter.
symbiont An organism participating in a relationship in which both species derive benefit.

Abbreviations

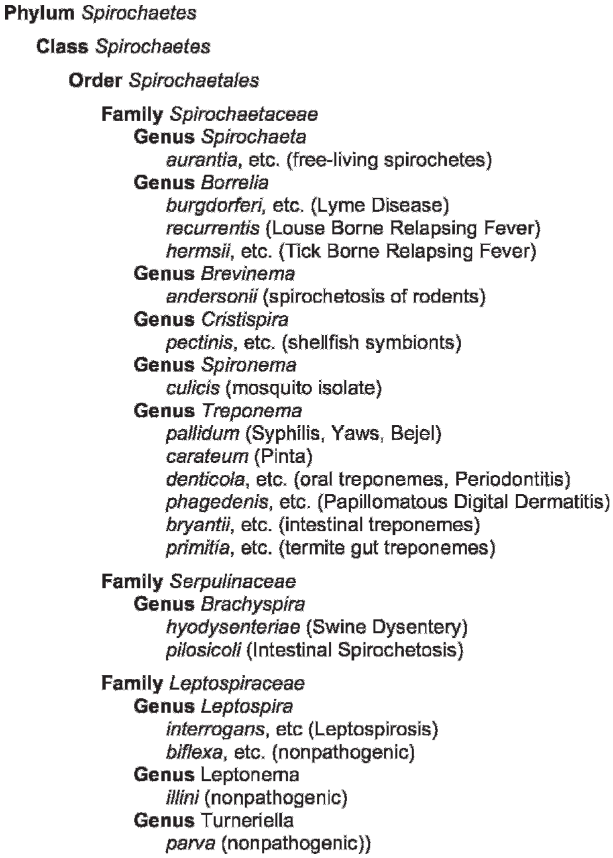
BSA	Bovine serum albumin
DHS	downstream homology sequence
EMJH	Ellinghausen–McCullough–Johnson–Harris
HisK	histidine kinase sensors
IS	insertion sequence
LBRF	louse-borne RF
LD	Lyme disease
Lig	*Leptospira* immunoglobulin-like repeat
LPS	lipopolysaccharide
MCPs	methyl-accepting chemotaxis proteins
Msp	major sheath protein
NADH	nicotinamide adenine dinucleotide
Omps	outer membrane proteins
PCR	polymerase chain reaction
PD	pocket depth
PDD	papillomatous digital dermatitis
RF	relapsing fever
TBRF	tick-borne RF
UHS	upstream homology sequence
VSH	virus of *Serpulina hyodysenteriae*

Defining Statement

Spirochetes are ancient bacteria that comprise one of the major phyla within the eubacterial kingdom. Their unique morphology and rotational motility are distinguishing features that allow rapid microscopic identification. Spirochetes are widely distributed in nature as free-living bacteria, as metabolic symbionts of insects, and as commensals and parasites of animals.

Overview

The spirochetes form one of the major phyla of the kingdom of Eubacteria. The depth of the spirochetal branch of the bacterial tree of life is indicated by the fact that phylum Spirochaetes has a single class and a single order. As shown in **Figure 1**, the order Spirochaetales is divided into three families, Spirochaetaceae, Serpulinaceae, and Leptospiraceae. The first family, Spirochaetaceae, includes a complex group of organisms that have adapted to diverse niches. At one extreme, there are a large number of free-living *Spirochaeta* organisms that can be cultivated from virtually any moist, nutrient-rich environment. At the other extreme is the obligate parasite, *T. pallidum*, which relies on the activities of a single animal host, man, for its survival and dissemination. In between these two extremes are the commensal, parasitic, and symbiotic organisms with life cycles involving insects, animals, or both. The second family, Serpulinaceae, contains a single genus, *Brachyspira*, and a more narrowly focused lifestyle involving residence in the lower intestinal tracts of animals. The third family,



Phylum *Spirochaetes*
Class *Spirochaetes*
Order *Spirochaetales*
Family *Spirochaetaceae*
Genus *Spirochaeta*
aurantia, etc. (free-living spirochetes)
Genus *Borrelia*
burgdorferi, etc. (Lyme Disease)
recurrentis (Louse Borne Relapsing Fever)
hermsii, etc. (Tick Borne Relapsing Fever)
Genus *Brevinema*
andersonii (spirochetosis of rodents)
Genus *Cristispira*
pectinis, etc. (shellfish symbionts)
Genus *Spironema*
culicis (mosquito isolate)
Genus *Treponema*
pallidum (Syphilis, Yaws, Bejel)
carateum (Pinta)
denticola, etc. (oral treponemes, Periodontitis)
phagedenis, etc. (Papillomatous Digital Dermatitis)
bryantii, etc. (intestinal treponemes)
primitia, etc. (termite gut treponemes)

Family *Serpulinaceae*
Genus *Brachyspira*
hyodysenteriae (Swine Dysentery)
pilosicoli (Intestinal Spirochetosis)

Family *Leptospiraceae*
Genus *Leptospira*
interrogans, etc (Leptospirosis)
biflexa, etc. (nonpathogenic)
Genus Leptonema
illini (nonpathogenic)
Genus Turneriella
parva (nonpathogenic))

Figure 1 Taxonomic organization of the spirochetes. Three families of spirochetes have been defined. Family Spirochaetaceae includes the free-living *Spirochaeta* spp., the parasitic *Borrelia*, and the commensal, parasitic, and symbiotic *Treponema* spp. Family Serpulinaceae are bacteria that colonize the lower intestinal tracts of mammals. Family Leptospiraceae includes both free-living nonpathogens and organisms that are able to invade animal reservoir hosts.

Leptospiraceae, includes both environmental saprophytes (e.g., *L. biflexa*) and animal parasites (e.g., *L. interrogans*) that cycle between bodies of freshwater and their preferred reservoir host.

Comparison of genome sizes indicates that life outside the host is much more genetically challenging than a life of host dependence. Free-living organisms such as *Spirochaeta aurantia and L. biflexa* have relatively large genomes relative to *Escherichia coli* (**Figure 2**). In contrast, adaptation of spirochetes to a commensal or parasitic lifestyle has resulted in genomic contraction. For example, *Leptospira borgpetersenii* and *L. interrogans* evolved from a common ancestor that had the ability to survive in both nature and the mammalian host, whereas *L. borgpetersenii* has become an obligate parasite of cattle that requires direct transmission from animal to animal. As a result, the *L. borgpetersenii* genome has become 16% smaller and remains in a process of decay, with 12% of its genes as nonfunctional pseudogenes. *Treponema denticola* has an intermediate-sized genome, perhaps related to the fact that although it is found only in animal hosts, it competes for nutrients in the complex oral microbial community. The *Borrelia* spp. and *T. pallidum* have the smallest genomes, with chromosomes only 1 Mb in size, which is consistent with their host dependence and lack of a free-living phase of their life cycle.

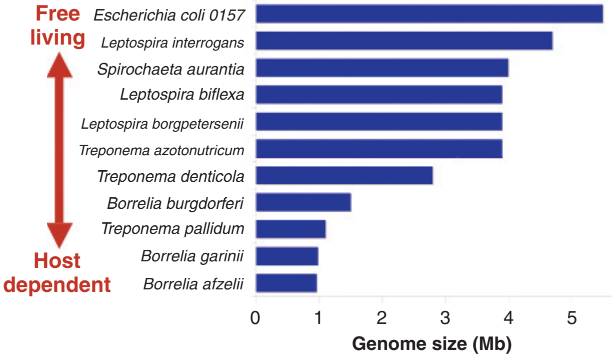


Figure 2 Comparative genome sizes of spirochetes. Free-living spirochetes, including *Leptospira* and *Spirochaeta* spp., have genomes that rival the size of *E. coli*. Host-dependent spirochetes, such as *T. pallidum* and *Borrelia* spp., have some of the smallest known genome sizes. Treponemes that live in the complex environments of the oral cavity (*T. denticola*) and termite gut (*T. azotonutricum*) have intermediate-sized genomes.

Spirochetes are defined by their unique morphology and rotational motility. Most spirochetes are helical coils – the one exception being *Borrelia burgdorferi*, which is actually a flat wave. Spirochetes are expert swimmers that are entertaining to watch by dark field microscopy. In low-viscosity liquids, spirochetes appear to spin in place. Increasing the viscosity by the addition of methylcellulose allows spirochetes to bore through the medium at a high rate of speed. The observer is quickly led to an understanding of how their screw-like movements would impart invasive properties to spirochetal pathogens. The organs of motility are flagella anchored near each end of the cell. Spirochete flagella are sometimes referred to as 'endoflagella' because they are subsurface structures, wrapping around the protoplasmic cell cylinder, as shown in **Figure 3**, instead of extending out beyond the surface of the cell as in all other flagellated bacteria. In at least one case, spirochete flagella also determine cell shape. *B. burgdorferi* mutants lacking the *flaB* flagellar filament protein are rod-shaped rather than wavy. Spirochetes differ in flagellar number and length. *Leptospira* have a single flagellum at each end of the cell that extends only a short distance along the length of the cell. In contrast, *Cristispira* spp. have bundles of over a 100 flagella at each end.

Chemotaxis allows bacteria to swim toward attractants, such as nutrients, and away from repellants by controlling the rotational direction of the flagellar motor. Flagella can rotate in a clockwise or counterclockwise direction. Sensory

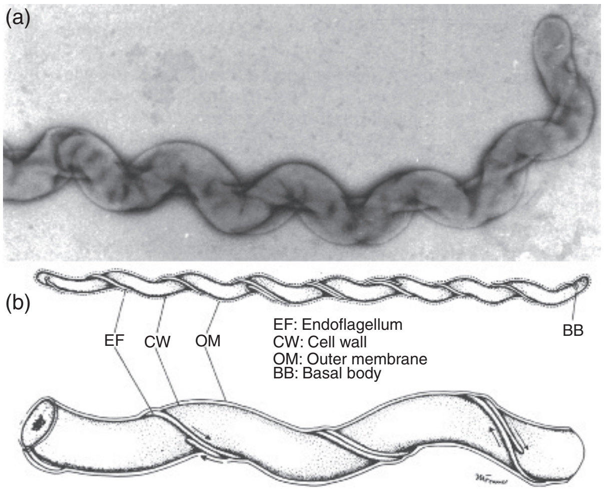


Figure 3 Spirochetal architecture. Spirochetes share a unique structure and motility strategy in which the endoflagella are inserted at opposite poles and wrap around the protoplasmic cylinder. (a) Electron micrograph of *Leptospira* showing a single endoflagellum at one end of the cell. (b) Schematic diagram showing endoflagellar location relative to the outer membrane and cell wall. Reproduced from Holt SC (1978) Anatomy and chemistry of spirochetes. *Microbiological Reviews* 42: 114–160.

proteins called methyl-accepting chemotaxis proteins (MCPs) control the directional switch in the flagellar motor. Clockwise rotation causes a cell to tumble (stop), counterclockwise rotation causes a cell to run (go). Spirochetes are unique in having flagella at each end. Effective spirochete movement involves flagellar rotation in the counterclockwise direction at the leading end and in the clockwise rotation at the trailing end. If the flagella at alternate ends are rotating in the same direction, spirochetes will flex in place rather than spin. It is not known how spirochetes coordinate the flagella at their alternate ends, or how MCPs orient spirochete movement. In any case, spirochetes are clearly adept at chemotaxis. For example, *B. burgdorferi* are able to find their way into a capillary tube containing *N*-acetylglucosamine, a sugar required for cell wall biosynthesis. Sensory proteins are used not only for chemotaxis but also for the regulation of gene expression. Spirochetes vary widely in terms of the number of sensory proteins they have. As mentioned previously, life outside the host is challenging and 'extroverts' (organisms with a free-living stage) have far more sensory proteins than 'introverts' that never leave the host. For example, *L. interrogans*, which lives both inside and outside a mammalian host, has 10 times the number of sensory proteins as *T. pallidum*, an obligate human parasite. **Figure 4** shows the correlation between lifestyle and numbers of sensory proteins.

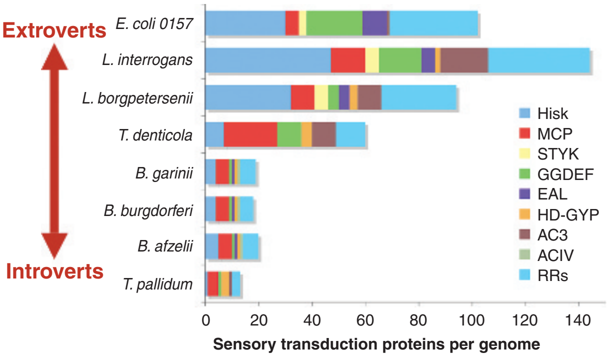


Figure 4 Comparison of spirochetal lifestyles and sensory transduction genes. Spirochetes have a wide variety of sensory transduction genes including histidine kinase sensors (HisK), methyl-accepting chemotaxis proteins (MCP), and so on. Spirochete 'extroverts' that live outside the host have a much greater number of sensory transduction proteins per genome than host-dependent 'introverts'.

The unique spirochetal architecture has both Gram-negative and Gram-positive features. Like Gram-negative bacteria, spirochetes are 'diderms', or double-membrane bacteria. However, the spirochetal outer membrane is much more fluid and labile than the outer membrane of Gram-negative organisms. In typical enteric Gram-negative bacteria, the outer membrane is supported by, and closely associated with, the underlying peptidoglycan cell wall. In contrast, the spirochetal cell wall is more closely associated with the inner, or cytoplasmic, membrane than the outer membrane (**Figure 5**), a feature of Gram-positive bacteria. Another important difference between the outer membranes

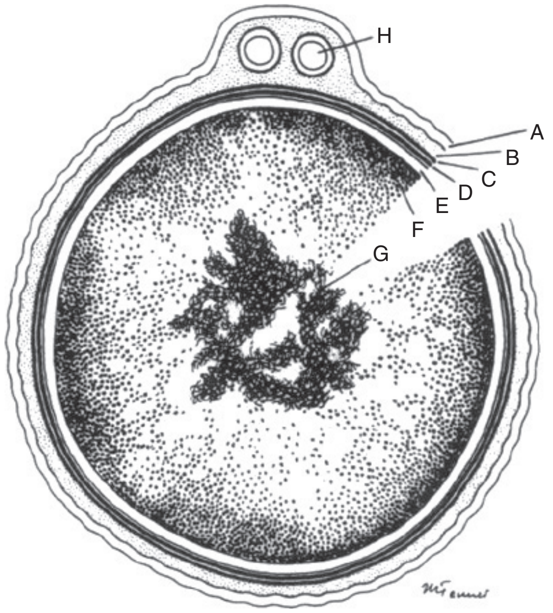


Figure 5 Spirochete cross-section. Elements of spirochetal architecture include (a) outer membrane; (b) periplasm; (c and d) peptidoglycan cell wall; (e) cytoplasmic membrane; (f) cytoplasm; (g) nuclear material; and (h) endoflagella. Note that the endoflagella are subsurface structures and that the cell wall is more closely associated with the cytoplasmic membrane than with the outer membrane. Reproduced from Holt SC (1978) Anatomy and chemistry of spirochetes. *Microbiological Reviews* 42: 114–160.

of most Gram-negative bacteria and those of treponemes and *Borrelia* is a lack of lipopolysaccharide (LPS). Leptospires have LPS, but there are significant structural differences between leptospiral and *E. coli* LPS such that human Toll-like receptor 4 is unable to bind to leptospiral LPS. The lack of recognizable LPS allows spirochetes to function as 'stealth pathogens' that are able to invade and persist in the bloodstream and in tissues of the body without detection by the early warning system of innate immunity.

Protein export pathways of spirochetes resemble those of other bacteria. The *Sec* pathway for exporting proteins with signal peptides across the cytoplasmic membrane is conserved. Genes encoding enzymes that process signal peptides are present in spirochete genomes, but their specificities are clearly unique because prediction algorithms such as Psort and LipoP frequently do not apply to spirochetal signal peptides. Computer recognition of signal peptides of spirochetal lipoproteins requires the development of spirochete-specific training sets and algorithms (e.g., SpLip). Upon reaching the periplasmic face of the cytoplasmic membrane, spirochetal lipoproteins are shuttled to the outer membrane via the Lol pathway. Here again, rules that apply for *E. coli* lipoproteins have been altered for spirochetal lipoproteins such that retention of spirochetal lipoproteins in the cytoplasmic membrane involves negatively charged amino acids after the N-terminal cysteine and export to the outer membrane is by default. Membrane fractionation and ultrastructure studies demonstrate three types of spirochetal outer membrane proteins (Omps), namely transmembrane porin-like molecules, lipoproteins, and peripheral (nonintegral) membrane proteins. All spirochetes have Omp85 homologues for assembly and insertion of Omps. Transmembrane Omps are required for transport functions and both transmembrane and surface lipoprotein Omps have been shown to be involved in host–pathogen interactions.

Treponema

The genus *Treponema* includes a broad diversity of parasitic and commensal species, most of which exist in complex bacterial communities. A notable exception is highly invasive obligate human pathogen, *T. pallidum*, subspecies *pallidum*, the agent of syphilis. *T. pallidum*, syphilis, and its history are covered in 'Sexually transmitted diseases' and 'Syphilis, historical' of the current edition of *Encyclopedia of Microbiology*. In passing, it should be mentioned that *T. pallidum* has two other subspecies and a sister species that cause nonvenereal skin infections of humans (see **Figure 1**). *T. pallidum* subspecies *pertenue* causes yaws, *T. pallidum* subspecies *endemicum* causes endemic syphilis (bejel), and *T. carateum* causes pinta. Another member of this species group is *Treponema paraluiscuniculi*, the agent of venereal spirochetosis of rabbits, which has an overall genome sequence similarity of 98.6–99.3% with *T. pallidum* subspecies *pallidum*. It should also be mentioned that a number of additional *Treponema* species have been isolated from the intestinal tracts of animals including cows (*Treponema bryantii* and *Treponema saccharophilum*) and pigs (*Treponema succinifaciens*). In this section, we will cover the oral treponemes, the organisms that cause papillomatous digital dermatitis (PDD) of cattle, and the termite gut treponemes that contribute to the digestion of cellulose.

Oral Treponemes

Oral treponemes were some of the first bacteria described in the writings and drawings of Antonie van Leeuwenhoek, the father of Microbiology. In 1676, when examining a dental plaque from the mouth of an old man, van Leeuwenhoek found "an unbelievably great company of living animalcules, a-swimming more nimbly than any I had ever seen up to this time. The biggest sort. . .bent their body into curves in going forwards. . ." Today, anyone with a dark field microscope can repeat van Leeuwenhoek's experiment. If the sample is taken from the periodontal space (located between the tooth and the gum) of a patient with gum disease, it is likely that spirochetes will be observed to be the predominant bacterial forms. A remarkable diversity of oral treponeme morphologies is present in the mouth, with a broad variety of diameters, lengths, wavelengths, amplitudes, and numbers of endoflagellae. Sizes range from 0.1 to 0.4 μm in diameter and from 5 to 20 μm in length.

The microbial community of the mouth, referred to as the oral 'microbiome', is estimated to include upward of 500 different bacterial species. Given the complex environment in which they live, it is not surprising that it has been relatively difficult to isolate and cultivate oral treponemes. Like most bacteria that live in the periodontal space, most oral treponemes are strict anaerobes. However, some treponemes, such as *T. denticola*, can tolerate low concentrations of oxygen. Treponemes are intrinsically resistant to rifampin, which makes it possible to use rifampin-containing culture medium to exclude other bacteria and select for treponemes. Ten species of oral treponemes have now been isolated, allowing more detailed studies of their morphologies and metabolic requirements. However, more detailed enumeration of oral treponeme diversity has become available through polymerase chain reaction (PCR)-based cloning and sequencing of bacterial 16S rRNA sequences. In one important oral microbiome study of healthy and periodontitis subjects, five novel treponemal species were found for every one that had been cultivated. Nearly 25% (49/215) of the new oral bacterial species discovered were treponemes! The oral diversity of phylum Spirochaetes is exceeded only by the phylum Firmicutes, which includes the streptococci. On the basis of these

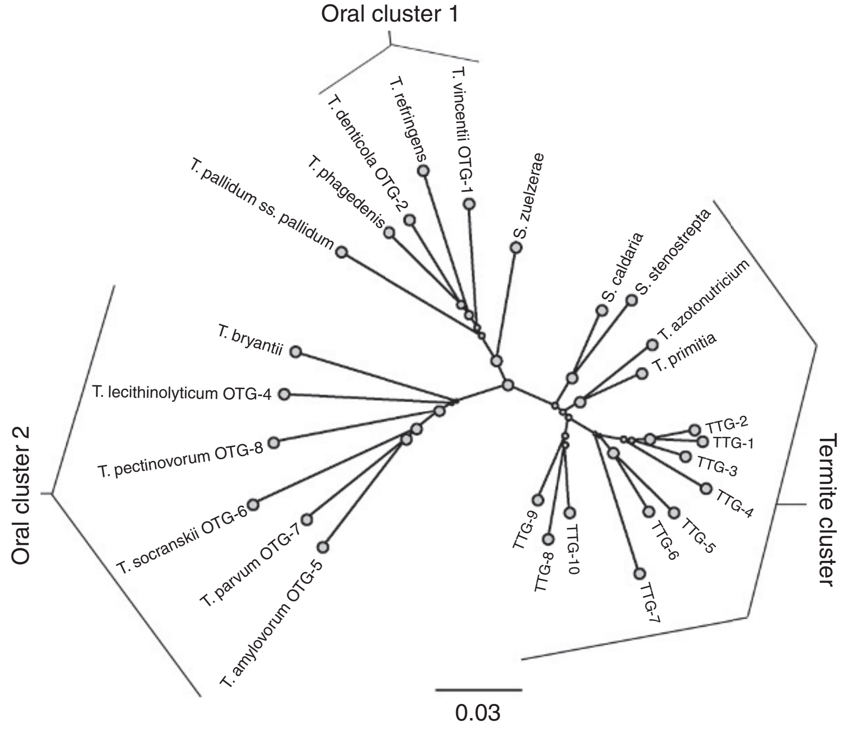

Oral cluster 1
T. vincentii OTG-1
T. refringens
T. denticola OTG-2
T. phagedenis
T. pallidum ss. pallidum
S. zuelzerae
S. caldaria
S. stenostrepta
T. azotonutricium
T. primitia
T. bryantii
T. lecithinolyticum OTG-4
T. pectinovorum OTG-8
T. socranskii OTG-6
T. parvum OTG-7
T. amylovorum OTG-5
Oral cluster 2
TTG-2
TTG-1
TTG-3
TTG-4
TTG-5
TTG-6
TTG-7
TTG-8
TTG-9
TTG-10
Termite cluster
0.03

Figure 6 Phylogenetic tree of the treponemes. Comparison of treponeme 16S rRNA sequences shows segregation into three relatedness clusters: two clusters of oral treponemes and a cluster of termite gut treponemes. Note that *T. pallidum*, the agent of syphilis, is related to the first cluster of oral treponemes.

molecular studies, ten phylogenetic groups of oral treponemes in two clusters have now been defined (**Figure 6**).

Several lines of evidence implicate treponemes as oral pathogens. Although treponemes can be found in small numbers in the mouths of healthy individuals, their numbers and diversity are strongly correlated with the severity of chronic and aggressive forms of periodontitis and their numbers are diminished with clinical treatment. Two species that have been associated with periodontitis are *T. denticola* and *Treponema lecithinolyticum*. Both gingivitis and periodontitis are extremely common inflammatory gum diseases. The distinction is that while gingivitis is reversible, periodontitis involves erosion of the dental ligament that attaches the tooth to the supporting bone at the base of the periodontal pocket (**Figure 7**). Peridontitis affects 50% of the US population over 30 years of age and is the leading cause of tooth loss. Although treponemes are typically found at the base of the periodontal pocket in association with other pathogenic organisms, such as *Porphyromonas gingivalis* and *Tannerella forsythia*, immunofluorescence microscopy shows that the treponemes are the most invasive organisms, typically invading the epithelial cells at the leading edge of the invasion process. Treponemes also appear to be involved in endodontal (root canal) infections; *Treponema maltophilum* DNA was detected in 50% of root canal samples using 16S rDNA-based PCR methods.

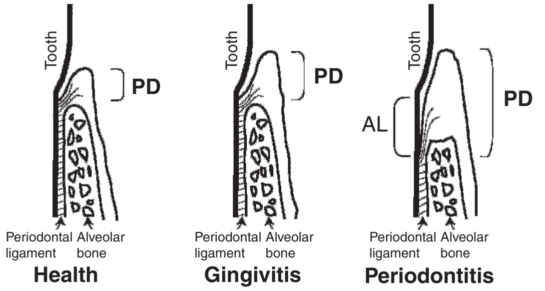

Tooth
PD
Periodontal ligament
Alveolar bone
Health
Tooth
PD
Periodontal ligament
Alveolar bone
Gingivitis
Tooth
AL
PD
Periodontal ligament
Alveolar bone
Periodontitis

Figure 7 Schematic representation of health, gingivitis, and periodontitis. The periodontal pocket depth (PD) is increased in gingivitis due to tissue swelling associated with inflammation. In periodontitis, the PD is further increased due to the loss of the tissue attachment to the root of the tooth (AL: attachment loss). Periodontitis is further characterized by the loss of supporting alveolar bone. Treponemes are typically found at the base of the periodontal pocket. Reproduced from Kinder-Haake S, *et al.* (2006) Periodontal diseases. In: Lamont *et al.* (eds.) *Oral Microbiology & Immunology*, ISBN-13: 9781555812621.

Several pathogenetic mechanisms have been identified by which oral treponemes cause disease. By virtue of their motility, chemotaxis, and narrow diameter, spirochetes are able to slip between epithelial cells and invade the subepithelial layers of the gum tissue. Although treponemes do not make Gram-negative LPSs, they do elaborate a variety of glycolipids and lipoproteins that stimulate innate inflammatory pathways. *T. denticola* expresses a serine protease, dentilisin, which digests host extracellular matrix proteins, including fibronectin, laminin, and fibrinogen. Dentilisin activates host matrix metalloproteinases, and together with dentilisin these enzymes serve to alter and eventually degrade the barriers that prevent invasion by other periodontal bacteria. Exposure to *T. denticola* causes cytoskeletal rearrangements that disrupt normal host cell functions. These cytotoxic effects are probably caused by the *T. denticola* release of Msp, the major sheath protein. Msp is a porin-like molecule that appears to insert into host cell membranes and trigger intracellular calcium fluxes, which are believed to damage epithelial cell barriers and impair the clearance of invaded bacteria by polymorphonuclear leukocytes.

PDD Treponemes

PDD is a polymicrobial infection of the soft tissue adjacent to the hoofs of cattle. Affected animals have painful ulcers referred to as heel warts or footwarts. Since it was first described in the 1970s, PDD has spread throughout the world, including most herds in the United States, and is now the leading cause of lameness in dairy cattle. The spread of PDD is related to industrial-scale dairy practices where cattle are continuously kept in barns or feedlots on moist surfaces and not allowed to graze. Cultures of PDD lesions show a mixed population of anaerobic bacteria including a number of spirochetes that are closely related to oral treponemes such as *T. denticola*, *Treponema medium*, and *Treponema vincentii.* Immunofluorescence studies of biopsies of PDD lesions reveal invasion of treponemes into the soft tissues of foot, not unlike the invasion of treponemes observed in the periodontium of the mouth.

Termite Gut Treponemes

Diversity

Although the volume of the termite hindgut can be as small as one microliter, the diversity of treponemal phylotypes and morphotypes within a single termite rivals that of the human mouth. Microscopy of the termite hindgut contents reveals treponemes ranging from 0.1 to 1 μm in diameter and from 3 to 100 μm in length, with a variety of cell wavelengths and amplitudes (**Figure 8**). Some of the larger forms can have 100 or more periplasmic flagella. Many termite gut treponemes are individual cells, whereas others are ectosymbionts of protozoa, functioning as their motility organelles. Recent metagenomic analysis of the total hindgut microbiota of arboreal wood-feeding *Nasutitermes* termites revealed that 68% of the genetic material was treponemal in origin. Termite treponemes form a distinct cluster within the treponeme phylogenetic tree (**Figure 6**). In addition to several named species, ten termite *Treponema* groups have been defined. The *Spirochaeta* species, *Spirochaeta stenostrepta* and *Spirochaeta caldaria*, were isolated from water as free-living organisms and named before their 16S sequences were known, but fall within the termite treponeme cluster and should be considered *Treponema* species likely to have been released from animals or insects.

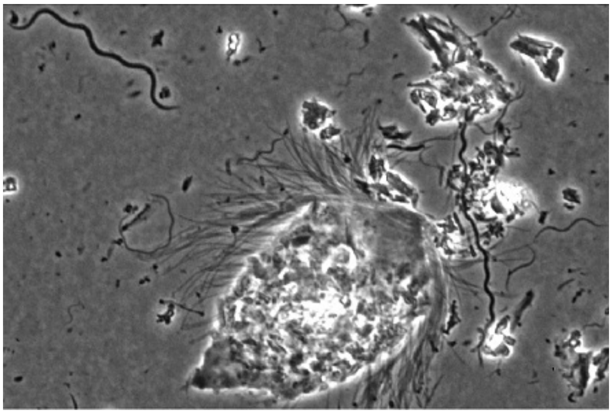


Figure 8 Phase contrast micrograph of termite gut treponemes. A variety of treponeme sizes and morphologies are present in the termite gut. Note the treponemal appendages attached to the hypermastigote protozoan, *Trichonympha Agilis*. Reproduced from Breznak JA (2006) In: Radolf JD and Lukehart SA (eds.) *Pathogenic Treponema: Molecular and Cellular Biology*, ISBN: 1-904455-10-7.

Metabolism

Unlike the commensal or pathogenic treponemes of the oral cavity, termite gut treponemes are symbionts, benefitting their termite hosts by contributing to the digestion of woody plant material. Like most host-dependent spirochetes, termite gut treponemes were difficult to isolate. Eventually, the first termite gut treponemes to be grown in pure culture were isolated from the California dampwood termite, *Zootermopsis angusticollis*, and assigned to the new species, *Treponema primitia. T. primitia* is an anaerobe, and was grown in sealed containers. In the process of working with the *T. primitia* cultures, it was discovered that a vacuum had developed in the headspace of the *T. primitia* cultures. This observation led to the finding that *T. primitia* could convert H_2 and CO_2 gases to acetate. Acetate was known to be a major source of energy and carbon for termites. The treponemes were found to reduce single carbon CO_2 to two-carbon acetate molecules via a well-known acetyl-CoA pathway, thus providing nutrients to the termite that would otherwise have escaped in a gaseous form. Treponemes were subsequently found to benefit termite metabolism in other ways. Cellulose is high in energy but relatively poor in nitrogen required for the formation of amino acids. A second species, the aptly named *Treponema azotonutricum*, was found to be able to convert significant amounts of atmospheric N2 to ammonium using its unique dinitrogenase reductase activity.

Until recently, it was not known to what extent termite gut treponemes participated in other aspects of wood polysaccharide digestion. Before CO_2 and H_2 are formed, cellulose and xylan must be hydrolyzed to hexose and pentose oligomers, respectively, which are in turn fermented to metabolic intermediates. The metagenomic analysis referred to previously revealed a rich diversity of treponemal cellulase and hemicellulase genes. Researchers demonstrated some of the predicted enzymatic activities in the termite gut lumen proteome. Genome sequencing efforts are currently under way to further elucidate the role of termite gut treponemes in cellulose digestion. The ability to convert cellulose to energy without releasing CO_2 has led to hopes that the enzymatic activities of termite gut treponemes can be harnessed for the production of green energy, in the form of termite farms, treponemal soups, or as recombinant organisms functionalized with termite gut treponeme genes.

Borrelia

Morphology and Metabolism

Borrelia spp. are divided into two large genetic groups: the relapsing fever (RF) *Borrelia* and the Lyme disease (LD)-related *Borrelia.* This article covers the RF Borrelia. The LD *Borrelia* are covered in 'Lyme disease' of the current edition of *Encyclopedia of Microbiology. Borrelia* vary from 8 to 30 μm in length and from 0.2 to 0.5 μm in width, with the RF *Borrelia* tending to be shorter and wider than the LD-related *Borrelia.* Borreliae exhibit the unique rotational motility of spirochetes powered by endoflagella. The RF borreliae have 15–30 endoflagella, whereas the LD-related borreliae have 7–11 endoflagella. Unlike the endoflagella of other spirochetes, the endoflagellae of *Borrelia* lack sheaths. The other distinguishing morphological characteristic of the *Borrelia* is the lack of cytoplasmic tubules.

Borrelia are obligate parasites with a life cycle that alternates between arthropod vectors and mammalian hosts. Despite their host dependence, many *Borrelia* spp. have been cultivated using nutritionally rich media, similar to tissue culture media, including many amino acids and vitamins. Glucose is required and is metabolized via the Embden–Meyerhof glycolytic pathway. *Borrelia* require exogenous N-acetylglucosamine for cell wall synthesis, presumably because this chitin component is constitutively available in ticks. Bovine serum albumin (BSA) is provided as a source of long-chain fatty acids for membrane biosynthesis. The bane of *Borrelia* researchers is the variable ability of different lots of BSA to support *Borrelia* growth. Although *Borrelia* make superoxide dismutase and are able to tolerate low levels of oxygen, they are oxygen sensitive. One possible explanation for the lot-to-lot variability of BSA is that polyunsaturated fatty acids supplied by certain lots of BSA appear to be the target of reactive oxygen species, resulting in damage to *Borrelia* membranes.

Epidemiology and Phylogeny

The *Borrelia* life cycle involves alternating parasitism of arthropod vectors and mammalian hosts. The aptly named *Borrelia recurrentis* is the only one of the RF *Borrelia* transmitted by the human body louse (*Pediculus humanus*) and is historically the most important of the RF *Borrelia.* Numerous plagues of RF have been recorded, dating back at least as far as the time of Hippocrates. Associations with war, famine, and displaced populations resulting in poverty and overcrowding were well known, but the specific association with body lice was not recognized until 1907. The twentieth century witnessed devastating epidemics of louse-borne RF (LBRF). In the aftermath of the Russian revolution, there were 13 million cases of LBRF in Russia and Eastern Europe, resulting in 5 million deaths. Because humans are the only mammalian host of LBRF and its vector, outbreaks can be effectively aborted by the treatment of clothes and bed linins with insecticides or simply by heating to at least 55 °C (130°F) for 5 min. LBRF has been eradicated everywhere except for isolated areas of Ethiopia and neighboring countries involved in war (Sudan, Eritrea, and Somalia).

Aside from *B. recurrentis*, all other RF *Borrelia* are transmitted by ticks and have nonhuman animal host reservoirs. These tick-borne forms of RF are considered endemic zoonoses and are found worldwide. Most (but not all) *Borrelia* causing tick-borne RF (TBRF) are transmitted by the *Argasidae* family of soft-body ticks, whereas the LD-related *Borrelia* are transmitted by the *Ixodidae* family of hard-body ticks. Because of the close relationship between TBRF *Borrelia* species and their tick vectors, the names of the *Borrelia* species derive from the species of tick vectors that transmit them: *Borrelia hermsii* is transmitted by *Ornithodoros hermsi*, *Borrelia parkeri* is transmitted by *Ornithodoros parkeri.*

The distinction between different types of ticks is important because their different feeding strategies dictate the circumstances under which humans are likely to encounter the *Borrelia* they carry. Soft-body ticks are nocturnal feeders that seek out sleeping animals by following carbon dioxide and temperature gradients. Although large in size, they have a painless bite and their soft bodies have a distensible stomach that allows them to feed rapidly (15–90 min), drop off, and disappear before being recognized. The large blood meal allows soft-body ticks to live for up to 15 years between feedings, while retaining viable *Borrelia* in their midgut. *Ornithodoros* species of soft-body ticks may transmit *Borrelia* to their progeny, a process referred to as 'transovarial transmission'. The frequency of transovarial transmission of *Borrelia* to tick progeny varies greatly between tick species. The frequency of transovarial transmission is high in *Ornithodoros turicata*, low in *O. hermsi*, and does not occur in *O. parkeri.*

Hard-body ticks seek out their blood meal during the day and feed for longer periods of time (typically days). The smaller stomach size also requires more frequent feedings. Blood meals are required for a hard-body tick to mature from larvae to nymph, from nymph to adult, and then for the adult to reproduce. Hard-body ticks are mentioned here because there are two notable exceptions to the rule that TBRF *Borrelia* are transmitted by soft-body ticks: *B. miyamotoi* is transmitted by *Ixodes* species (wood ticks) and *Borrelia lonestari* is transmitted by *Amblyomma americanum* (the lone star tick).

16S rRNA sequences of RF *Borrelia* spp. separate phylogenetically into the following three relatedness groups: Old World RF *Borrelia*, New World TBRF *Borrelia*, and the *B. hermsii*/*B. anserina* group (**Figure 9**). *B. hermsii* is the most common agent of human TBRF in North America and is endemic to the coniferous forests of the western United States and southern British Columbia from 3000 to 9000 feet in elevation. The incidence of TBRF peaks in July and August when vacationers visit rustic cabins in mountainous locations that are inaccessible during the winter. *B. hermsii* achieves high blood densities for prolonged periods of time in pine squirrels (*Tamiasciurus* spp.), which serves to facilitate transmission to other ticks. Chipmunks and some rodents may also become infected but with lower blood densities and for shorter periods of time than in pine squirrels. Two distinct genomic groups of *B. hermsii* have been described based on sequencing the intergenic spacer region between the 16S rRNA and ileT tRNA genes. The distributions of the two *B. hermsii* genomic groups overlap geographically, indicating that migratory animals, such as birds, may play a role in dissemination. *B. hermsii* has been found in the bloodstream of a dead owl, and is phylogenetically related to *B. anserina*, the agent of avian spirochetosis (**Figure 9**).

Other New World TBRF *Borrelia* differs from *B. hermsii* in their geographical distribution. *B. turicatae* occurs in the southwestern United States and northern Mexico. Although *B. turicatae* has not been isolated from humans, evidence strongly implicates it as the cause of TBRF in spelunkers in Texas. *B. parkeri* isolates from ticks in the coastal regions of California and Baja California have been implicated as a cause of human disease, but the evidence is circumstantial. Recently, the 16S sequence of a related *Borrelia* species was obtained from the argasid bat tick, *Carios kelleyi*, from an attic in Iowa. There is the potential for human disease given the close phylogenetic relationship with human pathogens, the cohabitation of *C. kelleyi* in homes and the willingness of *C. kelleyi* to feed on humans. *Borrelia coriaceae* is transmitted by soft-body *Ornithodoros* ticks and its reservoir in North America is the black-tailed deer. Human infection with *B. coriaceae* has not been described, but it is believed to cause abortion in cattle. *Borrelia mazzottii* and *Borrelia venezuelensis* have been described in Central and South America, but their 16S rRNA sequences and relatedness to other New World TBRF *Borrelia* are unknown.

Some New World TBRF species are transmitted by hard-body ticks. Like *B. burgdorferi*, *B. miyamotoi* is found in

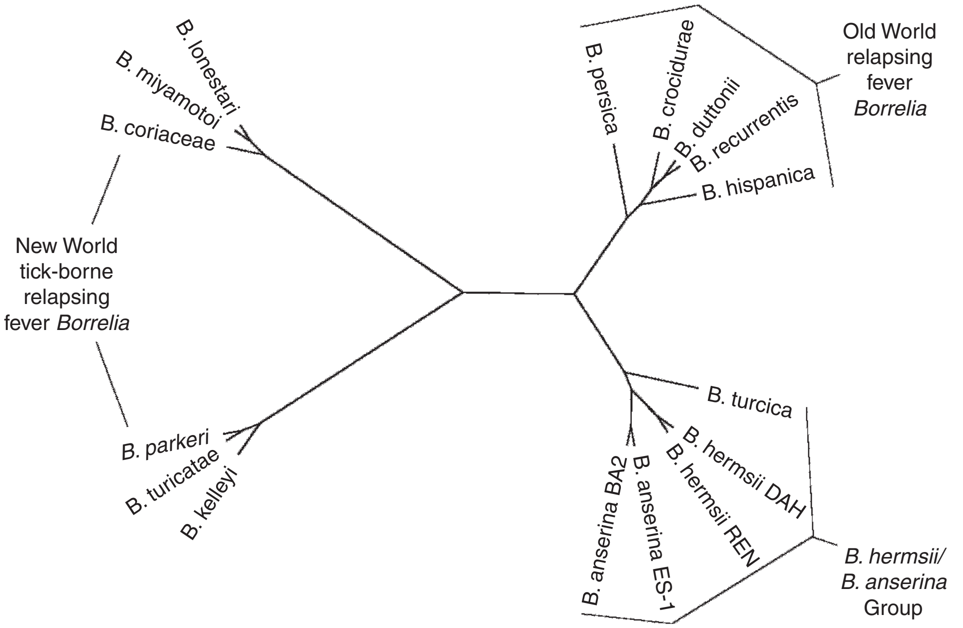


Figure 9 Phylogenetic tree of the relapsing fever *Borrelia*. 16S rRNA sequences of Old World *Borrelia* spp., including *B. recurrentis*, the agent of louse-borne relapsing fever, cluster in the lower right section of the tree. Sequences of New World *Borrelia* spp. cluster in the upper section of the tree. Sequences from *B. hermsii* and the bird-associated *B. anserina* cluster in the lower left section of the tree.

Ixodes ticks and *Peromyscus leucopus*, the white-footed mouse. *B. lonestari* is carried by *A. americanum*, the lone star tick, which is widely distributed in North America and is known to transmit ehrlichiosis and tularemia. The ability of *B. lonestari* to infect humans is unknown. Among Old World TBRF *Borrelia*, *Borrelia duttonii* is the species that is genetically most similar to *B. recurrentis*, and they probably share a common ancestor. *B. duttonii* and the related species, *Borrelia crocidurae*, are transmitted by soft-body ticks and are important causes of TBRF in Sub-Saharan Africa. A number of other Old World TBRF *Borrelia* have been described in the Middle East, Caucasus, and central Asia, but 16S rRNA sequences are available for only a couple of these species: *Borrelia persica* and *Borrelia hispanica*, found in Israel and Spain, respectively.

Molecular Pathogenesis and Disease

The molecular mechanisms of antigenic variation that are the hallmark of RF have been best described in *B. hermsii.* In the tick, the major *B. hermsii* surface protein is the variable tick protein, which presumably facilitates tick–spirochete interactions. In response to temperature changes during the blood meal, *B. hermsii* switches expression to the variable protein locus located on the expression plasmid. As the bacteria begin to reach high densities in the bloodstream of the infected animal, the host mounts an antibody response to the protein encoded by the gene in the variable protein expression locus. Clearance of bacteria by variable protein-specific antibody is eventually followed by the emergence of bacteria that have undergone a recombinational event on the expression plasmid involving the insertion of genes encoding any one of 12 variable small proteins or 15 variable large proteins. It had long been observed that there was a bias toward a patterned sequence of variable protein gene insertion events. Recently, an explanation for the pattern was explained by the upstream homology sequence (UHS) and downstream homology sequence (DHS) of the variable genes. The probability of a subsequent gene being inserted into the variable protein expression locus was related to the homology of its UHS with the gene currently in the locus and the distance from the end of the new gene to its DHS.

A programed succession of surface proteins enables RF *Borrelia* to repeatedly emerge at high levels in the bloodstream (**Figure 10**). The ability to repeatedly emerge into the bloodstream is advantageous to the bacteria, because it favors acquisition by blood-feeding arthropods. However, such a high density of bacteria is very hazardous to their animal host, because it evokes such an intense immune response to the foreign antigens. Bouts of LBRF and TBRF differ in their intensity and in the number of relapses. LBRF tends to recur less often, but the episodes are much more severe, with a mortality rate of 4–40%. After a typical incubation period of 7 days, patients experience sudden onset of fever, rigors, headache, muscle pain, and lethargy. In LBRF, most patients have liver and spleen enlargement, while cough and symptoms of meningitis are common. Nerve palsies, paralysis, seizures, and coma may occur in severe cases. The most common causes of death in LBRF are arrhythmias of the heart, brain hemorrhage, and liver failure. LBRF during pregnancy frequently results in miscarriage. The mortality rate in TBRF is typically much lower, that is, 2–5%. Nevertheless, TBRF due to *B. turicate*, *B. duttonii*, and *B. crocidurae* are frequently associated with debilitating neurologic symptoms not seen with other forms of TBRF.

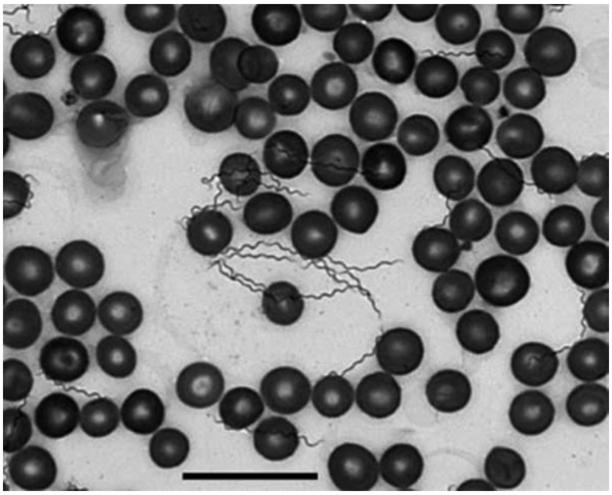


Figure 10 Micrograph of blood containing relapsing fever *Borrelia*. Variation in surface antigens enables relapsing fever *Borrelia* to reach high levels in the bloodstream, often achieving densities as high as 10^6–10^7 bacteria per milliliter. Spirochetes appear as dark wavy forms. Reproduced from Figure 1 in Schwan et al., Tick-borne Relapsing Fever Caused by Borrelia hermsii, Montana. *Emerging Infections Diseases* 2003; 9(9): 1151–4.

RF *Borrelia* are susceptible to a broad range of antibiotics. LBRF can be successfully treated with a single dose of tetracycline. β-Lactam antibiotics such as penicillin are typically avoided in LBRF because they may result in the sudden lysis of large amounts of bacterial antigens, which can precipitate the Jarisch–Herxheimer reaction, a paradoxical worsening of symptoms with severe chills, fever, and potentially life-threatening shock. Patients should be observed for 2°h after the initiation of antibiotics in case there is a need for resuscitation with intravenous fluids.

Brachyspira

The second major grouping within the order Spirochaetales are the *Brachyspira*, which are intestinal spirochetes classified within the family, Serpulinaceae. *Brachyspira* are large, loosely coiled spirochetes ranging in size from 2 to 13 μm in length and from 0.2 to 0.4 μm in width. *Brachyspira* are able to grow under strict anaerobic conditions, but small amounts of oxygen can increase growth efficiency. The *nox* gene, encoding NADH (nicotinamide adenine dinucleotide) oxidase, is required for oxygen tolerance. Inactivation of the *nox* gene increases oxygen sensitivity 100-fold. *Brachyspira* are cultivated anaerobically on blood agar at 37 °C and selective media are typically used for primary isolation of organisms from stool specimens.

The *Brachyspira* have undergone a series of changes in nomenclature. The isolation of the swine dysentery agent was originally described in the early 1970s and referred to as *Treponema hyodysenteriae*. In the 1990s, DNA–DNA hybridization and partial 16S sequence data indicated that the *T. hyodysenteriae* organism had little genetic relatedness to the treponemes and was assigned its own genus, *Serpula*, which was quickly reclassified as *Serpulina* to avoid confusion with a previously named fungal genus. However, *Serpulina* eventually gave way to *Brachyspira* when it was realized that *Serpulina hyodysenteriae* was related to *Brachyspira aalborgi*, which had been isolated from humans with intestinal spirochetosis in Aalborg, Denmark, in the early 1980s. The prefix Brachy, deriving from the Greek word for 'short', was used as a descriptive term because the Danish isolates were only 2–6 μm in length.

Brachyspira hyodysenteriae is an important worldwide problem for the pig industry. Outbreaks with mortality rates of up to 50% occur in naive herds. The infection is a true dysentery, causing inflammatory and hemorrhagic disease of the colon. *B. hyodysenteriae* is *β*-hemolytic on sheep blood agar and hemolysins are believed to be important virulence factors. The organism is difficult, but not impossible, to eradicate from farms. In Scandinavia, where the use of antibiotics is strictly controlled, few herds are infected by *B. hyodysenteriae*. In most countries, antibiotic supplementation of feed is used to suppress the *B. hyodysenteriae* problem, and infection rates are often over 30%. However, antibiotic resistance is growing and new strategies for prevention and control of *B. hyodysenteriae* infection are urgently needed.

The genus *Brachyspira* is now populated with a number of commensal and pathogenic species, which have been isolated from the intestinal tracts of a variety of animal hosts. Species with predilections for pigs, humans, and birds are clustered on a phylogenetic tree from their 16S sequences (**Figure 11**). *Brachyspira suanatina* is the name proposed for an organism that is related to, but genetically distinct from, *B. hyodysenteriae* by 16S rRNA sequence analysis. *B. suanatina* has been isolated from both pigs and mallard ducks, is *β*-hemolytic, and can cause disease in experimentally infected pigs. *B. intermedia* is a third pig isolate found in the same genetic cluster with *B. hyodysenteriae* and *B. suanatina*, and may cause disease under certain circumstances. Nondysenteric porcine diarrhea due to intestinal spirochetosis has been linked to *B. pilosicoli*, which has also been associated with disease in chickens and humans (see below). *B. innocens* and *B. murdochii* are considered to be commensals occasionally isolated from healthy pigs.

Brachyspira species are also important in the poultry industry. Diarrhea and egg production problems in chickens have been attributed to *B. alvinipulli*, *B. intermedia*, and *B. pilosicoli*. As in pigs, *B. innocens* and *B. murdochii*, and a

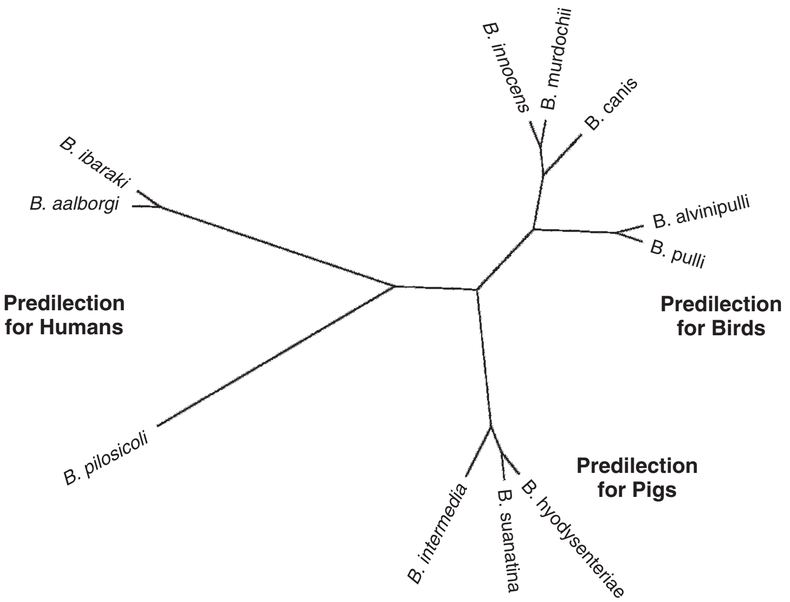


Figure 11 Phylogenetic tree of the *Brachyspira*. Relatedness tree of 16S rRNA sequences of *Brachyspira* spp., including *B. hyodysenteriae*, the agent of swine dysentery. *B. aalborgi* and *B. pilosicoli* cause intestinal spirochetosis in humans in developed and developing countries, respectively.

third species, *B. pulli*, appear to be nonpathogenic for chickens. Chronic watery diarrhea owing to human intestinal spirochetosis has been linked to two species, *B. aalborgi* and *B. pilosicoli*, with the latter being associated with intestinal disease in pigs and chickens. High prevalence rates of *B. pilosicoli* carriage have been found in aboriginal populations living in poor sanitary conditions with high levels of animal exposure. In contrast, *B. aalborgi* occurs more frequently in developed countries, typically in AIDS patients with chronic diarrhea. The pathogenic potential of *Brachyspira* for humans is controversial. Biopsies show palisades of *Brachyspira* lining the surface of colonic epithelial cells, which is likely to impair function (**Figure 12**). *B. pilosicoli* is associated with watery diarrhea and has been isolated from the bloodstream of sick patients.

Efforts are ongoing to sequence the genomes of *B. hyodysenteriae* (3.2 Mb) and *B. pilosicoli* (2.45 Mb). The overall structure of the *B. hyodysenteriae* genome is likely to be relatively unstable due to the presence of the interesting Virus of *S. hyodysenteriae* (VSH-1) prophage. Upon induction with mitomycin, VSH-1 functions as a general transduction agent, transferring random 7.5 kb fragments of *B. hyodysenteriae* DNA between bacteria. *B. hyodysenteriae* is an attractive organism for research on microbial pathogenesis because of the availability of techniques for targeted gene inactivation. In 1992, researchers at the University of Utrecht reported the first successful homologous recombination in a spirochete, inactivating the *B. hyodysenteriae tlyA* gene encoding a putative hemolysin. The *tlyA* mutant had reduced hemolytic activity on blood agar plates, and virulence was attenuated in mouse challenge studies. Subsequently, a number of additional candidate hemolysin genes have been identified, and it is likely that *B. hyodysenteriae* β-hemolytic activity is multifactorial. As in other spirochetes, the *Brachyspira* outer membrane is decorated with membrane proteins. The nomenclature proposed for *Brachyspira* membrane proteins includes the initials of the species name (Bh for *B. hyodysenteriae*), the type of protein (lp for lipoprotein and mp for membrane protein), and the predicted molecular mass of the mature protein. So the family of *B. hyodysenteriae* 29.7 kDa lipoproteins formerly referred to as BmpB and BlpA should now be referred to as Bhlp29.7a, Bhlp29.7b, and so on. Bhlp29.7a has been shown to be lipidated, is a component of the *B. hyodysenteriae* outer membrane proteome, and is recognized by sera from infected pigs, indicating expression during infection. Omps expressed during infection are of great interest as potential vaccines and serodiagnostic antigens.

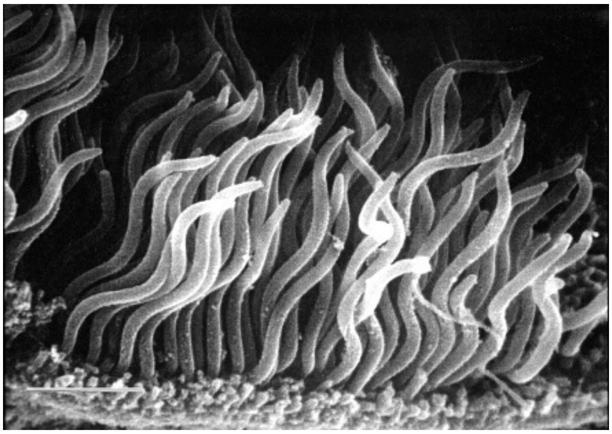


Figure 12 Scanning electron micrograph showing palisades of *Brachyspira* exhibiting end-on attachment to the luminal surface of colonic epithelial cells. Marker bar = 2 μm. Reproduced from Hampson DJ and Stanton TB (eds.) (1997) *Intestinal Spirochaetes in Domestic Animals and Humans*, ISBN: 0-85199-140-8.

Leptospira

Morphology and Metabolism

Leptospira derives from the Greek *leptos* (thin) and Latin *spira* (coiled). Aptly named, the leptospires are among the thinnest bacteria known: a mere 0.1 μm in diameter and 6–12 μm in length (**Figure 13**). Leptospires are right-handed helices, with 18 or more coils per cell, frequently forming hooks at one or both ends. Hooks at both ends gave rise to the species name *L. biflexa*, and a hook at one end was believed to look like a question mark, leading to the name *L. interrogans*. The hooks are due to a single endoflagellum at each end of the cell. In liquids, viable leptospires are continuously in motion. In semisolid (0.2% agarose) conditions, leptospires can be observed by dark field microscopy to remain motionless for periods of time, with occasional corkscrew-like movements. This resting state may, in part, explain the ability of leptospires to persist in the environment. Most leptospires are able to remain motile for months in distilled water, and their survival can be significantly prolonged by addition of a substrate such as agarose.

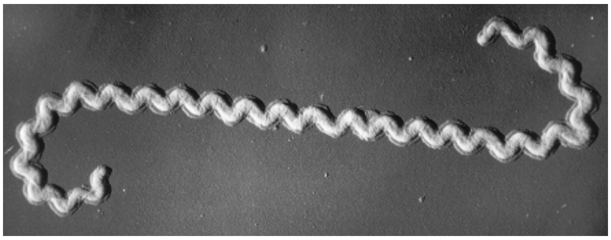


Figure 13 Transmission electron micrograph of *Leptospira* sp. showing characteristic helical morphology and a single endoflagellum at each end of the cell. Magnification ~×30,000. Shadowed electron micrograph obtained by Annabella Chang and used with permission from Ben Adler, Microbiology Department, Monash University, Australia.

Several different leptospiral growth media have been developed. The standard culture medium is Ellinghausen–McCullough–Johnson–Harris (EMJH) medium, which provides long-chain fatty acids in the form of tween (polysorbate) as an energy and carbon source, several divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}), iron, and vitamins (thiamin and cobalamin). EMJH medium contains BSA, which is believed to function by preventing fatty acid oxidation, which is toxic for spirochetes, and by providing additional trace nutrients. BSA is expensive and batch-to-batch variability in its ability to support leptospiral growth is a major problem. Serum is not required for EMJH medium but is often added to promote growth. Another problem with BSA-containing media is that due to prior concerns, many countries, including the United States, require any bovine products such as BSA to be autoclaved before import. A non-BSA containing leptospiral medium that can be used as an alternative transport medium is modified Kortoff medium, which consists of peptone, salts, and 8–10% heat-inactivated slightly hemolyzed rabbit serum. The optimum growth temperature is 30 °C.

Phylogeny

A number of different *Leptospira* species have been described with a range of pathogenic potentials (**Figure 14**). On one end of the spectrum are the nonpathogenic saprophytic species, including *L. biflexa* and *Leptospira wolbachii*, which are unable to cause infection. On the other end of the spectrum are the pathogens, including *L. interrogans*, which are able to produce lethal infection in a variety of mammals, including humans. Species with intermediate pathogenicity, such as *Leptospira fainei*, can be isolated from clinical specimens, but cause minimal or no disease. Leptospires can also be classified serologically, and over 250 different named serovars have been described. Serovars are classified into one of 28 different serogroups on the basis of antigenic cross-reactivity. Serovar specificity appears to be driven by the carbohydrate structure of LPS side chains, a dominant antigen on the leptospiral surface. There is limited correlation between the genetic and serologic classification systems, with serologically identical strains occurring in multiple species.

The phylogenetic and antigenic diversity of *Leptospira* species reflects their ability to adapt to a variety of different environmental niches. Leptospires have been isolated from most animal species (including, reptiles and amphibians) and natural bodies of freshwater wherever the effort has been made. Presumably, leptospires represent an ancient branch of the bacterial family tree that has coevolved with vertebrates. In reservoir host animals, leptospires have developed a unique commensalist strategy. Organisms with the capacity to infect animals typically reside in the lumen of the proximal renal tubule and are shed into the environment in the urine. The fluid within the proximal tubule is a nutritionally rich filtrate of serum and is yet an immunologically protected site; kidney sections of infected rats show little

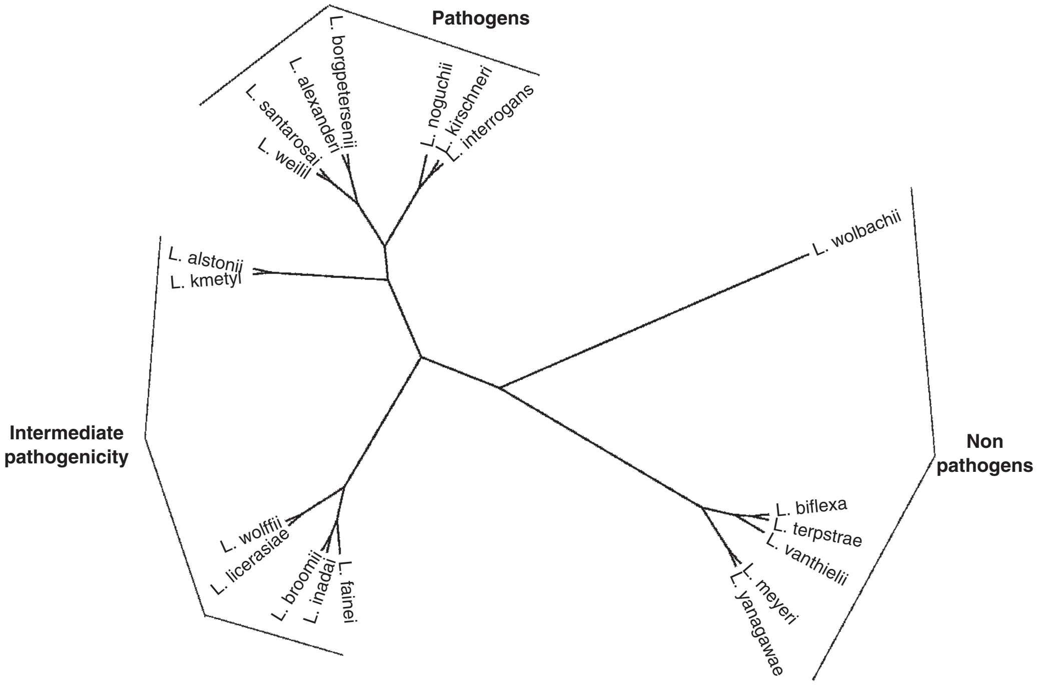


Figure 14 Phylogenetic tree of the *Leptospira*. Comparison of leptospiral 16s rRNA sequences shows segregation into three relatedness clusters: Nonpathogens (lower left), pathogens (lower right), and organisms with intermediate pathogenicity (upper section).

or no inflammatory reaction surrounding infected tubules. By not subjecting their host to any detrimental effects, this arrangement effectively affords leptospires a 'free ride' for the life of the animal host. The life cycle of the organism is completed when organisms released into the environment encounter a new host through adhesion, vascular invasion, and dissemination to the kidney.

Genome sequences provide insight into differences between the various leptospiral lifestyles. The free-living saprophytic nonpathogen, *L. biflexa*, has a genome size of 3.96 Mb, with a relatively high coding density, and an abundance of signal transduction genes, enabling it to respond to the unpredictable environmental stresses found outside the host. In contrast, *L. interrogans* has a biphasic lifestyle and seems equally at home in the aquatic environment and in the mammalian host. The 63% of *L. interrogans* genes shared with *L. biflexa* consist of essential housekeeping genes and genes important in survival outside the host. The remaining 37% of *L. interrogans* genes are presumed to be important for life within the mammalian host. On the other end of the spectrum is *L. borgpetersenii*, which has evolved into an obligate parasite of cattle. Infection occurs through direct contact with carrier animals, *L. borgpetersenii* has limited survival outside the host, and is difficult to culture. This host dependence is reflected in the erosion of the *L. borgpetersenii* genome; many of its genes are lost or inactivated by mutations or transposon insertions. *L. borgpetersenii* has only about half the signal transduction genes that *L. biflexa* has, confirming the 'locked-in' nature of its host dependency.

Pathogenesis

To acquire the ability to invade and colonize the mammalian host, *L. interrogans* has acquired a large array of novel genes. Some of these pathogen-specific genes are known to encode Omps such as the porin, OmpL1, and a number of lipoproteins, some of which are involved in host–pathogen interactions. An essential host–pathogen interaction that distinguishes leptospiral pathogens from saprophytes is serum resistance. Leptospiral serum resistance is mediated, at least in part, by LenA, an outer membrane lipoprotein found exclusively in leptospiral pathogens. LenA binds Factor H, a complement regulatory protein that prevents the alternative pathway of complement from damaging host cell membranes. Leptospires (and other spirochetes) coat their surfaces with Factor H to avoid the bactericidal effects of complement.

Pathogenic leptospires coat their surfaces with additional host factors using proteins belonging to the Lig (*Leptospira* immunoglobulin-like repeat) family. Leptospiral pathogens, but not the saprophytes, have between one and three Lig proteins. Ligs are very large (112–220 kDa) proteins containing a series of 12–13 immunoglobulin-like repeats, some of which mediate high affinity binding to multiple host proteins, including fibronectin and fibrinogen. Interactions with host proteins are facilitated by the induction of Lig expression in response to levels of osmolarity (300 mOsm) found in host tissues. Lig expression by leptospires grown in EMJH medium, which has low osmolarity (67 mOsm), is poor. Addition of salt (or any other osmotically active molecule) to EMJH medium rapidly induces Lig expression. In this way, leptospires in aquatic environments are saved the metabolic expense of not expressing Lig proteins until they are needed.

Acquisition of virulence genes was essential in the evolution of leptospires from free-living to pathogenic organisms. Genes appear to have been horizontally transferred from a variety of sources. For example, the major outer membrane lipoprotein, LipL32, is highly conserved among leptospiral pathogens and is believed to mediate interactions with extracellular matrix proteins of the host. The *lipL32* gene does not occur in the nonpathogens, its closest homologue is found in the marine bacterium *Pseudoalteromonas tunicata.* Horizontal genetic transfer also occurs between leptospiral pathogens; 20% of *ompL1* genes are mosaics containing fragments of multiple leptospiral lineages. However, permissiveness for gene acquisition is a double-edged sword – the genomes of leptospiral pathogens have much higher numbers of insertion sequence (IS) elements than the nonpathogens. The IS elements contain transposon genes that, once they infect the genome, mediate IS element proliferation and gene disruption. IS elements appear to be a major mechanism of genome erosion in *L. borgpetersenii.* Transposons are now being put to good use in leptospiral research – leptospiral pathogens had been much more difficult to transform than the nonpathogens, which had been a major impediment in leptospiral pathogenesis research. Now, however, the mariner tranposon has been found to be useful for manipulating the genome of leptospiral pathogens – hundreds of single-gene knockout mutations have been generated in *L. interrogans* strains. It is hoped that testing these mutants in animal models will lead to the identification of new leptospiral virulence genes and vaccines.

Epidemiology and Disease

Leptospirosis epidemiology has traditionally been carried out by serotyping isolates or examining the serologic response of infected patients. However, serologic approaches are fraught with problems, including the frequent observation that patient's antibody responses may not be specific for the infecting serovar. Genetic tools provide more accurate molecular approaches for tracking the epidemiology of leptospirosis. 16S sequencing can be used for species identification and is less cumbersome than DNA–DNA hybridization. Differentiation of strains has been performed by multilocus sequence typing using PCR primers for 11 housekeeping genes scattered across the leptospiral genome. Approaches such as these reveal that rat-associated strains of *L. interrogans* are frequently the cause of leptospirosis outbreaks in urban settings. Rats are found wherever people live, and wherever the studies have been carried out, urban rats are found to have a high leptospirosis carriage rate in their kidneys. The prevalence of leptospiral carriage among rats is probably the reason that leptospirosis is the most widespread zoonosis known. Leptospirosis occurs less frequently in Westernized countries because housing standards tend to exclude rats from human living spaces. However, in developing countries with poor housing standards, leptospirosis outbreaks occur regularly in urban settings after heavy rainfall and flooding.

Leptospirosis infections range in severity from self-limited flu-like illness to multiorgan system failure and death. After an incubation period of 5–14 days, there is the onset of fever, myalgia, headache, abdominal pain, nausea, and vomiting. In tropical regions where leptospirosis typically occurs, these early nonspecific symptoms can be confused with dengue fever or malaria. When observed, conjunctival suffusion (scleral redness without discharge) can be a distinguishing sign of leptospirosis. During this initial septicemic phase, spirochetes can be recovered from the blood and spinal fluid. Formation of agglutinating antibody leads to the clearance of organisms and, in milder cases, to a temporary resolution of symptoms. However, a second, immune phase of the disease may follow, with milder fever, headache, and vomiting. In more severe infections, the initial phase progresses rapidly to jaundice and renal failure, known as Weil's syndrome, with a mortality rate of 10%. The renal failure due to leptospirosis is a unique form of kidney dysfunction associated with high urine output and low serum potassium levels. At this stage, complications can be avoided with the replacement of fluids and electrolytes. If, on the other hand, dehydration occurs and renal failure ensues, access to peritoneal or hemodialysis is essential for survival. Certain strains of *L. interrogans* cause acute lung involvement with shortness of breath due to airspace hemorrhage and a much higher mortality rate of 50%.

Pathogenic leptospires are highly susceptible to common antibiotics including doxycycline and ampicillin and it is likely that antibiotic therapy given at the first signs of infection would significantly reduce morbidity and mortality. However, currently available diagnostic tests have relatively low sensitivity during early infection and patient populations at highest risk typically have poor access to medical care. Recent studies show that most patients with early infection have antibodies to the Lig proteins. What is needed is a diagnostic test that is portable, easy to use, does not require electricity, and has a long shelf life at room temperature. Whole-cell vaccines are used widely in domestic animals, including dogs, pigs, and cattle. A similar vaccine has been found to be effective in humans, but is generally not available because of concerns regarding side effects and a relatively short duration of immunity. A preventative approach for adventure travelers participating in water sports in areas with a history of leptospirosis is weekly doxycycline, which has been shown to be effective in US soldiers undergoing jungle training in Panama. Doxycycline is not appropriate for children or pregnant women and may cause photosensitivity or gastrointestinal side effects. An alternative approach recommended by some travel experts is weekly azithromycin, which has a better safety profile, but has not been rigorously tested for efficacy.

Conclusions

Spirochetes are widely distributed in nature as free-living bacteria, as metabolic symbionts of insects, and as commensals and parasites of animals. *Spirochaeta* spp. isolated from natural bodies of water are related by 16S rRNA sequence analysis to treponemes found in the oral cavity and in the digestive tracts of termites. *Borrelia* spp. also have the ability to colonize the digestive tracts of insects, in this case ticks and lice, which serve as vectors for transmission to animal host reservoirs. *Brachyspira* spp. colonize digestive tracts of animals either as commensals or as parasites. *Leptospira* spp. exist as free-living organisms or cycle between the aquatic environment and animal host reservoirs via their renal tubules. The diversity of spirochete lifestyles demonstrates the functional versatility of their unique morphology and mechanism of motility.

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Staphylococcus

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Glossary

biofilm Sessile microbial communities consisting of an accumulated mass of bacteria, extracellular matrix molecules, and secreted bacterial products that aid in adherence to biopolymers and other solid surfaces.

capsule Polysaccharide outermost layer of the cell. Production imparts a viscous slimy look to colonies.

cell wall One of the outer layers of the bacterial cell that protects the cell from osmotic perturbations and provides mechanical protection to the fragile cellular membrane.

endonuclease Enzymes that have specific binding sites of varying complexities at which they sever phosphodiester bonds of DNA.

MSCRAMM (Microbial surface components recognizing adhesive matrix molecules) Proteins involved in the establishment of infection via the initial attachment of bacteria to host molecules such as elastin, collagen, fibrinogen, and fibronectin.

plasmid Autonomously replicating small extrachromosomal DNA molecules.

superantigen Any of a number of proteins that elicit a massive T-cell receptor Vβ-restricted primary response.

Abbreviations

CDC	Centers for Disease Control
CHEF	clamped homogeneous electrophoretic field
cMRSA	community-acquired MRSA
CoNS	coagulase-negative staphylococci
IFN-γ	interferon-γ
MHC II	major histocompatibility complex class II
MRSA	methicillin-resistant *Staphylococcus aureus*
MSCRAMM	microbial surface components recognizing adhesive matrix molecule
NCBI	National Center for Biotechnology Information
PIA	production of a slime substance
PVL	Panton-Valentine leukocidin
RFLP	restriction fragment length polymorphism
SE	staphylococcal enterotoxin
SSSS	staphylococcal scalded-skin syndrome

Defining Statement

The staphylococci are important bacterial pathogens that can infect both animals and humans and are responsible for numerous hospital- and community-acquired infections yearly. Staphylococcal infections result in a significant burden both economically and clinically due to several factors, including increasing antibiotic resistance and lack of effective vaccines.

Taxonomy

The genus *Staphylococcus* is defined in *Bergey's Manual of Determinative Bacteriology* as a member of the family *Micrococcaceae.* According to the most recent approved list from *Bergey's Manual* (8th ed.), there are presently 41 recognized species of staphylococci (**Table 1**), 18 of which are indigenous to or have been shown to colonize humans; the remaining species have been isolated from

Table 1 Currently recognized staphylococcal species

S. arlettae	*S. aureus*[a]
S. auricularis[a]	*S. capitis*[a]
S. caprae[a]	*S. carnosus*
S. caseolyticus	*S. chromogenes*
S. cohnii[a]	*S. condimenti*
S. delphini	*S. epidermidis*[a]
S. equorum	*S. felis*
S. fleurettii	*S. gallinarum*
S. haemoyticus[a]	*S. hominis*[a]
S. hyicus	*S. intermedius*
S. kloosii	*S. lentus*
S. lugdunensis[a]	*S. lutrae*
S. muscae	*S. nepalensis*
S. pasteuri[a]	*S. pettenkoferi*[a]
S. piscifermentans	*S. pseudintermedius*
S. pulvereri	*S. saccharolyticus*[a]
S. saprophyticus	*S. schleiferi*[a]
S. sciuri[a]	*S. simiae*
S. simulans[a]	*S. succinus*
S. vitulinus[a]	*S. warneri*[a]
S. xylosus[a]	

[a] Denotes species that are either indigenous to humans or have been found to colonize humans.

various animal (including one primate species), plant, or food specimens. Staphylococci are Gram-positive cocci 0.7–1.2 μm in size that occur singly and in pairs in liquid media and in clusters when grown on solid media. Over the years, they have been characterized by their variety of colonial, morphological, and biochemical activities that have resulted in description of several biotypes of variable stability. They are aerobic or facultatively anaerobic, catalase-positive, and capable of generating energy by respiratory and fermentative pathways. These organisms are nutritionally fastidious with complex nitrogen requirements. Most species require several amino acids, vitamins (thiamine and niacin), and uracil (to grow anaerobically) for growth. In complex, nutritionally complete growth media, the organism is able to grow at generation times of $\leq$20 min, a rate comparable to that of *E. coli*.

At the molecular level, the genus can be distinguished from other members of the *Micrococcaceae* by the low GC content of its DNA which ranges from 30 to 38%, the presence of teichoic acid in their cell wall, and their ability to tolerate extremely low water activities. *Staphyloccocus aureus* is routinely cultured at a_w of 0.88 (15% NaCl) and has been reported to grow at water activity levels as low as 0.83 (saturated NaCl solution). Anaerobically, tolerance to low a_w is less with the limit at 0.90. The normal habitat of these organisms is the skin, skin glands, and mucous membranes of warm-blooded animals. However, staphylococci can be isolated from a variety of sources that include soil, dust, air, water, and food and dairy products. As a result they present a food and water public health hazard as well as an infection risk.

Cellular Structure

Cell Wall

The cell wall of *Staphylococcus* is a thick, electron-dense structure that provides great mechanical support to the cell. It is composed of a giant polymer consisting of peptidoglycan complexed with teichoic acid and other surface proteins described elsewhere in this article. The cell wall is a heteropolymer consisting of glycan chains cross-linked through short peptides. The repeating unit in the glycan backbone is β-1,4-*N*-acetylglucosamine and *N*-acetylmuramic acid (muramic acid). About 60% of the *N*-acetylmuramic acid residues are O-acetylated. The high level of O-acetylation makes the staphylococcal cell wall resistant to lysozyme digestion, therefore making this genus unique from most other bacteria. In fact, lysis of the cell wall under laboratory conditions is only efficient when the staphylococcal endopeptidase, lysostaphin, is added to cultured cells. Lysostaphin is produced by *S. simulans* and specifically cleaves the pentaglycine crossbridges found in the staphylococcal peptidoglycan. In *S. aureus*, adjacent polypeptides are cross-linked by pentaglycine crossbridges between the ε-amino of lysine and C-terminal D-alanine, whereas in other species, the composition of the crossbridge is variable. The carboxy group of muramic acid is substituted by an oligopeptide that contains alternating L- and D-amino acids (L-alanine, D-glutamine, L-lysine, D-alanine, D-alanine). The teichoic acid component is linked to the D-alanine component of the mucopeptide by α- or β-glycosidic linkage through *N*-acetyl-D-glucosamine. In *S. aureus*, the teichoic acid backbone is ribitol based whereas in *S. epidermidis* it is glycerol based. In other species, glycerol teichoic acids are more common than their ribitol counterparts. These highly charged immunogenic cell wall components have been found to play a role in *S. aureus* nasal colonization, biofilm formation, and susceptibility to vancomycin and other glycopeptides.

Capsule

Capsule production has been shown to occur *in vivo* and *in vitro* for both *S. epidermidis* and *S. aureus*. For *S. epidermidis*, there have been at least three different capsular polysaccharide types identified in the literature. In contrast, 11 capsular polysaccharide serotypes have been described for the highly virulent species, *S. aureus*. The most common occurring serotypes among clinical isolates are types 5 and 8. The main components of the capsules are *N*-acetylaminouronic acids and *N*-acetylfucosamine. The genes for capsule production have been identified and are located in a single operon. The production of a capsule renders the staphylococci resistant to host defenses such as opsonization and phagocytosis. However, antibodies to capsular polysaccharides can neutralize the antiphagocytic properties of the capsule and opsonize the cell for phagocytosis. Opsonization has made the capsule a prime target in the search for an effective staphylococcal vaccine.

Several staphylococcal species, particularly *S. epidermidis* and *S. aureus*, have been shown to produce biofilms, and capsule production has been shown to be of particular importance during biofilm formation by aiding in adherence to biomaterials such as indwelling medical devices. A trademark of staphylococcal biofilm formation is the production of a slime substance (PIA), which is a polysaccharide composed of β-1,6-linked *N*-acetylglucosamines with partially deacetylated residues giving them a positive charge. Once bacterial cells are within this slime, they are protected from the host's immune defenses and are resistant to other treatments such as antibiotics. PIA is not the only component that has been shown to contribute to biofilm production under certain conditions, and research in this area has become very popular in the past several years. *S. epidermidis* is the primary cause of catheter-associated staphylococcal infections and is a primary producer of biofilms. *S. aureus* biofilms have also been shown in patients with diseases such as osteomyelitis and endocarditis. Biofilm production in the staphylococci has been shown to be multifactorial and appears to be a bacterial survival mechanism when cells are exposed to sublethal levels of antibiotics and/or other stressful environmental conditions (i.e., limited nutrients, changes in temperature, oxygen limitation). It is also important to note that large phenotypic variations in biofilm production exist, with some isolates incapable of biofilm formation regardless of culture condition and others being hyperproducers under a specific set of conditions. Continued focus on understanding all of the components involved will give us more insight into chronic staphylococcal infections, especially those involving medical devices, and may lead to more efficient treatment of these diseases.

Molecular Structure

Genome

Presently, the genomes of 18 different members of the genus *Staphylococcus* have been completely sequenced. This number includes 14 strains of *S. aureus*, 2 strains of *S. epidermidis*, 1 strain of *S. haemolyticus*, and 1 of *S. saprophyticus*. The genomic data for each of these projects are freely available on the World Wide Web at the National Center for Biotechnology Information (NCBI) website.

In all cases, the genome is circular and ranges from 2.49 Mb (*S. epidermidis*) to 2.9 Mb pairs (*S. aureus* strains). **Table 2** shows a summary of the information obtained from the 18 completed staphylococcal genome projects. Consistent with the *Micrococcaceae* family, all 18 of the isolates have a low GC content (~33%). The sequenced strains also exhibit a high amount of diversity within this genus, with some species and strains having acquired resistance to the antibiotics methicillin and/or vancomycin and some containing extrachromosomal elements called plasmids that most often contain additional genes that can contribute to pathogenesis. Among the sequenced isolates, the number of plasmids present varies from 0 to 3 and the size of these elements varies as well. Genetic and sequence data available indicate that in addition to the normal complement of housekeeping genes, the chromosome contains many accessory genetic elements that are not necessary for growth under laboratory conditions.

Table 2 Completed staphylococcal genome project

Organism	*Genome size*	*Plasmid(s) size*	*%GC*	*# of predicted genes*	*MRSA/VRSA*
S. aureus strain MW2	2.82 Mb	N/A	32.82%	2632	MRSA
S. aureus strain Mu50	2.9 Mb	25.1 kb	32.84%	2748	MRSA
S. aureus strain N315	2.84 Mb	24.6 kb	32.8%	2623	MRSA
S. aureus strain NCTC8325	2.82 Mb	N/A	32.86%	2892	N/A
S. aureus strain RF122	2.74 Mb	N/A	32.77%	2589	MRSA
S. aureus strain COL	2.81 Mb	4.44 kb	32.81%	2787	MRSA
S. aureus strain MRSA252	2.9 Mb	N/A	32.8%	2744	MRSA
S. aureus strain MSSA476	2.79 Mb	N/A	32.85%	2619	N/A
S. aureus strain USA300 – FPR3757	2.91 Mb	37 kb, 4.4 kb, 3.13 kb	32.69%	2691	MRSA
S. aureus strain JH1	2.9 Mb	30.4 kb	32.0%	2870	MRSA, VRSA
S. aureus strain JH9	2.9 Mb	30.4 kb	32.9%	2816	MRSA, VRSA
S. aureus strain Newman	2.9 Mb	N/A	32%	2687	N/A
S. aureus strain Mu3	2.9 Mb	N/A	32%	2776	MRSA, VRSA
S. aureus strain USA300 TCH1516	2.9 Mb	27.0 kb	32%	2802	MRSA
S. epidermidis strain ATCC12228	2.49 Mb	N/A	32.09%	2419	MRSA
S. epidermidis strain RF62A	2.64 Mb	27.3 kb	32.14%	2665	MRSA
S. haemolyticus strain JCSC1435	2.68 Mb	N/A	32.79%	2678	MRSA
S. saprophyticus strain ATCC15305	2.57 Mb	38.5 kb, 16.3 kb	33.18%	2514	MRSA

In *S. aureus* strains, pathogenicity islands SaPI1 and SaPI2 have been described and have been shown to encode virulence factors such as the toxic shock syndrome toxin gene, staphylococcal enterotoxin B, and as well as resistance to methicillin. In strains that do not contain SaPI1 or SaPI2 there are no allelic counterparts for these genes. The genes and overall genomic organization of *S. aureus* bear a striking resemblance to the genome of *Bacillus subtilis* such that the organism has been called a morphologically degenerate form of *Bacillus*. In addition, the genomic organization of the *S. aureus* strains sequenced thus far has been shown to be highly similar. Specifically, there is a genomic 'backbone' common to all *S. aureus* strains containing varying numbers of genes that may contribute to antibiotic resistance, tissue tropism, and virulence. Comparison of the two sequenced *S. epidermidis* strains showed that the overall genomic organization is similar, with two relatively small areas of inversion apparent when the two genomes are aligned to each other at the nucleotide level. **Figure 1** shows alignments at the nucleotide level for two representative *S. aureus* strains as well as the two *S. epidermidis* strains. Alignment of *S. haemolyticus* and *S. saprophyticus* to *S. aureus*, *S. epidermidis*, or to one another showed little or no conservation of gene order. However, at the level of protein content, members of the genus are highly similar and this is illustrated graphically in **Figure 2**. Specifically, when the translated sequences for several of the sequenced staphylococcal genomes are compared (at a minimum cutoff level of >= 40% similarity) to one another using *S. aureus* NCTC8325 as the reference strain (since this strain has the largest number of predicted open reading frames), the level of protein similarity is 61.4% across the entire group. Of course, the level of similarity is slightly higher when the same type of comparison is done within species (85–92%) but the overall similarity at the genus level is still highly significant. For perspective, the same comparison done with *S. aureus* NCTC8325 and *B. subtilis* showed only 59% overall protein similarity.

In addition to large pathogenicity islands the staphylococcal genome (especially *S. aureus*) typically contains multiple instances of transposable elements such as *Tn551*, insertion sequences IS256, IS257, IS1181, and others. *S. aureus Tn551* and its close relative *Tn917* have been extensively utilized to generate knockout mutations in staphylococci, many of which have been mapped and localized near other genes. The primary strain of *S. aureus* used for genetic manipulation and gene discovery is NCTC8325 and its derivatives. Also known as PS47, this is the propagating strain for the typing bacteriophage ø47 and is a member of phage group III. It is routinely used to generate batches of bacteriophage used for typing purposes and is lysogenized by three temperate phage: ø11, ø12, and ø13. A derivative (8325-4) that has been cured of all demonstrable phage was originally developed as the prototype strain whose genome was used to build a circular map based on genetic and physical parameters. With the advancements in whole-genome sequencing technology this map is no longer the primary resource used for mapping and typing experiments. However, in the absence of sufficient genomic information, this type of mapping is still used to examine new *S. aureus* isolates and to determine basic lineage information for these isolates.

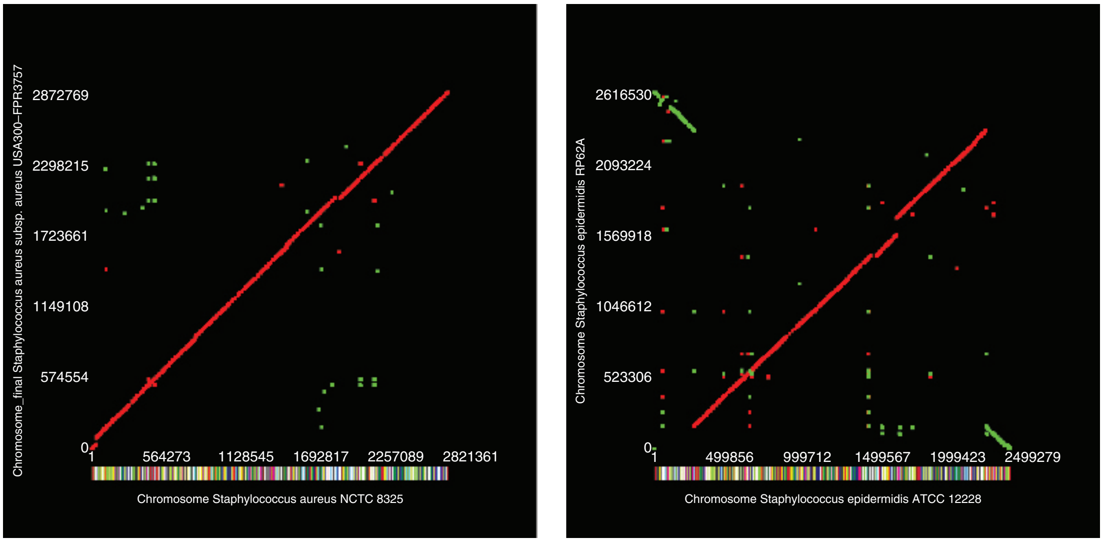

Figure 1 The left panel shows alignment of *S. aureus* strains NCTC8325 and USA300 at the nucleotide level. The solid red line indicates that the two strains are virtually identical in genomic organization. Right panel shows the alignment of *S. epidermidis* strains ATCC12228 and RP62A. The break in the red line and the shorter green lines show that although the two strains are overall highly similar in organization, there are some differences. Differences are believed to be due to the presence or absence of such things as bacteriophage and pathogenecity islands.

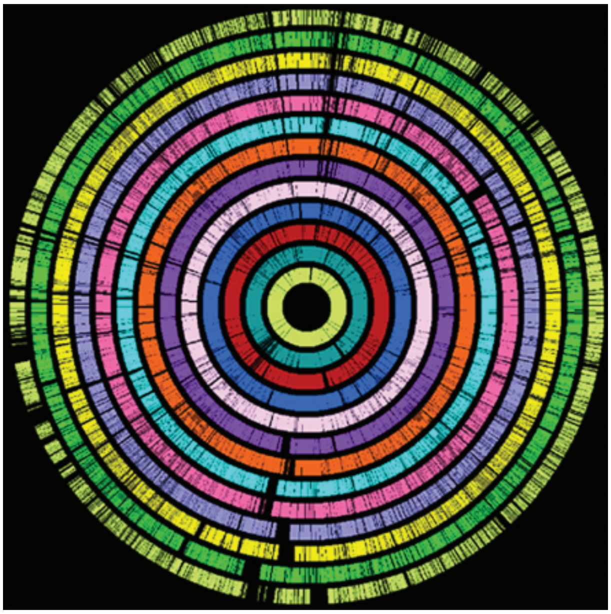

Figure 2 Comparison of proteins encoded within 13 members of the genus *Staphylococcus*. Completed genomes used in the above comparison are listed starting with the outermost circle: *S. aureus* strain NCTC8325, *S. aureus* strain COL, *S. aureus* strain USA300-FPR3757, *S. aureus* strain MW2, *S. aureus* strain Mu50, *S. aureus* strain MSSA476, *S. aureus* strain N315, *S. aureus* strain MRSA252, *S. aureus* strain RF122, *S. haemolyticus* strain JCSC1435, *S. epidermidis* strain ATCC12228 and *S. epidermidis* strain RP62A. Using NCTC8325 as the reference strain resulted in a total number of 2892 predicted proteins used for the comparision. Of the 2892 proteins used, 1775 were found to be present in all other genomes, with 2756 present in at least another genome and only 136 not found in any other of the comparison genomes.

Plasmids

As mentioned above, the staphylococci are also endowed with a generous array of plasmids ranging in size from a few kilobases to 40–50 kb. In fact, there are typically one or more plasmids found in most staphylococcal clinical isolates, including both *S. aureus* and the coagulase-negative staphylococci. The inability of two plasamids to coexist in a single host indicates that they have the same replication control functions and are incompatible and assigned to the same incompatibility group (*inc* group). Thus far, there are 15 recognized incompatibility groups (*inc* 1–15), but the replication characteristics of some staphylococcal plasmids remain undetermined and they are still unclassified. The 15 *inc* groups have been further divided into three main classes (I, II, and III) with a fourth class that contains the pSK639 family of plasmids. The four groups are based on both physical and genetic organization as well as functional characteristics. Some staphylococcal plasmids are cryptic but most carry resistance determinants and some carry other virulence factors such as toxins. Group I plasmids are relatively small plasmids (typically <5 kb) that replicate by the rolling circle mechanism. They are of a low copy number (10–60 copies per cell) and usually do not contain any transposons or mobility factors. The pSK639-type plasmids (group IV) are of intermediate size (8 kb range) in comparison to group I and II plasmids. The prototype plasmid for this group, pSK639, was originally isolated from *S. epidermidis* and was later found to be in some strains of *S. aureus*. pSK639-like plasmids carry only one resistance determinant and may harbor IS elements and/or transposons as well. The origin of replication appears to be similar to the theta replication system and unlike the group I plasmids they typically contain mobility factors. Group II plasmids are larger in size than the pSK639 group and are also known to carry multiple resistance factors. They range in size from 15 to 40 kb and have low copy number (~5 copies per cell). Along with the capability of encoding for several resistance factors on a single plasmid, they also contain transposable elements and like the pSK group replicate via theta mode. Group II plasmids generally confer resistance to β-lactam antibiotics and heavy metals and are also the group responsible for conferring methicillin resistance. Plasmids in group III are typically of the largest size (30–60 kb) and are conjugative in nature. They are found in both coagulase-negative staphylococci and *S. aureus* and tend to carry a number of resistance determinants. The replication region is similar in sequence to the group II plasmids and similarly they contain multiple copies of IS elements and transposons. Although conjugation of these staphylococcal plasmids is not completely understood, it is known to require cell-to-cell contact on a solid surface; also, there is no pilus as described in conjugation of *E. coli* plasmids. Vancomycin resistance at both high and intermediate levels has been shown to be encoded on group III plasmids, and there is evidence suggesting that the resistance marker and the plasmid have been acquired through transfer from the enterococci. It is widely held that plasmids are exchanged among staphylococci, enterococci, streptococci, and bacilli, accounting for the presence of the same or similar plasmids in each genus.

Staphylococcal Bacteriophage

S. aureus was among the first organisms used to demonstrate the existence of bacteriophage. In fact, intensive study of staphylococcal bacteriophage has led to the establishment of a complex method of bacteriophage typing of disease isolates identified in epidemiological investigation. A panel of 21 bacteriophage called the International Typing Series of Bacteriophages is used to define strain types by specific patterns of susceptibility to these typing phage. Phage typing led to the identification of five strain groups (I–IV and miscellaneous). Most pathogenic strains belong to groups II and III, but all phage groups are capable of causing disease. More recently, phage typing has lost favor because of the finding that the standard bacteriophage-propagating strains carry temperate bacteriophage that are distinct from the typing phage they propagate. The phage preparations derived from these strains are therefore mixtures of several bacteriophage. In fact, virtually all strains of *S. aureus* are lysogenized by at least one bacteriophage and many carry multiple temperate phage in their genome. Some of the lysogenizing phage carry additional genes that they bring in to the recipient strain and alter its phenotype by inserting into the genome at specific attachment sites located within genes. Both negative conversion (the inactivation of expressed genes (e.g., lipase and β-toxin)) and positive conversion (the introduction of newly expressed genes (staphylococcal enterotoxin A and staphylokinase)) are common occurrences in staphylococci. Temperate bacteriophage have been shown to alter the restriction pattern of the genome by introducing additional restriction sites that produce restriction fragment length polymorphisms (RFLPs) when analyzed by clamped homogeneous electrophoretic field (CHEF) gel electrophoresis. The creation of additional sites has caused ambiguities and difficulties in establishing clonal derivations in many epidemiological investigations of disease outbreaks. Phage typing of the staphylococci has also been made more difficult because not all methicillin-resistant *S. aureus* (MRSA) isolates come under the defined phage categories. Recently, studies have been performed to identify new phage to recognize this group as well as to identify alternative typing methods that may be more reliable for all kinds of isolates.

Bacteriophage are also thought to play a major role in the transmission of virulence factors and in the occurrence of genetic rearrangements. They are responsible for the only naturally occurring means of genetic exchange in the staphylococci. The first reports of transduction in staphylococci occurred in 1960 and concerned the transmission of resistance to penicillin. Since then many detailed studies of the transductional transmission of genes have been published. Most staphylococcal bacteriophage are generalized transducers, capable of transferring ~40–45 kb of DNA (a headfull) to a suitable recipient strain. DNA is packaged from PAC sites that are randomly dispersed around the genome.

Bacteriophage also play a role in transformation after induction of competence by Ca^{2+} treatment. Both plasmid and chromosomal DNA can be transferred by transformation, albeit at low frequency. In order for the transforming DNA to be recombined, it is necessary that the recipient strain be lysogenized by a serogroup B bacteriophage (there are 12 serogroupings). Other procedures, such as electroporation, enjoy limited effectiveness for plasmid transfer, but are not useful for transfer of chromosomal genes.

Coagulase-Negative Versus Coagulase-Positive Staphylococci

The primary players in human infection are actually *S. epidermidis*, *S. aureus*, and, to a lesser degree, *S. saprophyticus*. These species are basically identical except for the ability of *S. aureus* to produce the fibrinogen clotting substance coagulase. The coagulase-negative species, *S. epidermidis*, is largely commensal and can be isolated in high numbers from all areas of human skin. It was proposed by Baird–Parker that coagulase distinguished pathogenic from nonpathogenic species and this differentiation was used until the mid-1970s. However, recent evidence has shown that coagulase-negative staphylococci (CoNS) are a major cause of wound infections and infections caused by foreign bodies including intravascular catheters, peritoneal dialysis catheters, prosthetic heart valves, joint prostheses, pacemaker electrodes, and fluid shunt systems. Therefore, coagulase is no longer considered an exclusive indicator of pathogenicity but is still used as a method for species identification.

S. epidermidis and *S. saprophyticus* are ultimately distinguished from *S. aureus* based on the lack of coagulase activity. Of the staphylococci that are of medical importance, *S. aureus* is the only one that is coagulase-positive. Two other staphylococcal species (*S. hyicus* and *S. intermedius*) are also coagulase-positive but are rarely isolated from human infections. *S. aureus* also differs from the other clinically relevant members of this genus in that it is β-hemolytic on blood agar and forms large, yellow-pigmented colonies upon growth.

In a clinical setting it is imperative that the particular species involved is correctly identified as this plays a role in treatment options. All staphylococci are Gram-positive, coccoid, and grow as clusters in culture. *S. saprophyticus* grow as small, nonhemolytic, white colonies on blood agar and are isolated almost exclusively from urinary tract infections in young, sexually active females. *S. epidermidis* is the most abundant organism found on the skin of humans and is characteristically seen as nonhemolytic, white colonies on blood agar plates. Only half of the 32 CoNS have been found in humans and this group can be further divided based on whether they are resistant or sensitive to novobiocin. *S. epidermidis* is novobiocin-susceptible while *S. saprophyticus* is novobiocin-resistant. Clinical manifestations of coagulase-negative staphylococci are more subtle and nonspecific as compared to *S. aureus* and are also less likely to be fatal. However, with *S. epidermidis* in particular, these infections typically involve indwelling medical devices and are most often chronic. Indeed, the large majority of CoNS infections are nosocomial and strains isolated from humans typically produce an inducible β-lactamase, with 60–80% of them resistant to methicillin.

Pathogenesis and Disease

Before discussing staphylococcal pathogenesis it is important to note the difference between colonization and infection. Colonization can be defined as the presence of an organism in or on a particular body site in the absence of signs and symptoms of disease. Colonizing organisms can typically cause disease if they spread to a different site on the same individual (i.e., from skin to bloodstream) or are transferred to a more susceptible host. Infection is defined as the ability to isolate a replicating organism from a disease site within the host. Infection may result in immediate signs and symptoms or may be clinically unapparent. However, infection by an organism does eventually result in some level of inflammation within the host. Interestingly, although a host can become infected as soon as a pathogen invades, in many cases colonization precedes infection.

Carriage

Staphylococci are one of the major groups of organisms that inhabit the skin of mammals. Usually, several different strains are found on the same host. They may be present as transient contaminants, short-term replicating residents, or as long-term colonizers that persist for long periods. The majority of species are opportunistic pathogens that become infectious when the skin or mucous membranes are compromised by trauma, inoculation by needle, or direct implantation of medical devices (foreign bodies). Staphylococci are then able to attach, colonize, and produce toxic substances that destroy host tissues.

As stated previously, the majority of staphylococcal disease is due to infection with the coagulase-positive species *S. aureus*, which is considered an opportunistic pathogen because it is not uncommon for healthy individuals to carry the organism either persistently (10–35% of population) or intermittently (20–75%) in the anterior nares. Although colonization is not uncommon, it is also not ubiquitous among humans. In fact, recent studies have shown that 5–50% of people never carry *S. aureus*. In addition, the higher carriage rates are often related to the level of exposure; with healthcare workers often more likely to be colonized than individuals in the general population. Most often, carriers of *S. aureus* show no signs of disease; however, this organism can cause severe, even life-threatening disease under certain circumstances.

Disease Types

Species of *Staphylococcus* are the leading cause of nosocomial infection. Each year about 2 million hospitalizations result in nosocomial infection and ~50% are due to *S. aureus* and *S. epidermidis*. Community-acquired infections have a similar incidence rate. A predisposing condition has been the acquisition of multiple drug resistance by *S. aureus*, which has caused the incidence of nosocomial and community-acquired infection to increase steadily since the 1960s.

The primary mode of pathogenesis for *S. epidermidis* is via colonization of biomedical devices such as indwelling catheters as a result of biofilm formation on the surface of these biomaterials. Although *S. aureus* causes a wide variety of pyogenic and toxin-mediated diseases, *S. epidermidis* rarely causes pyogenic disease in the immunocompetent host and there is very little evidence to suggest that it is responsible for toxin-mediated disease. Outside of indwelling medical device infection, *S. epidermidis* is primarily seen in intravenous drug users and immunocompromised patients such as premature newborns and individuals undergoing immunosuppressive therapy. The clinical manifestations associated with these groups are right-sided endocarditis and septicemia, respectively. Unlike *S. aureus* infections, there are typically no fulminant signs of infection and the clinical course is therefore more chronic in nature.

S. aureus is a more highly pathogenic organism and has been isolated from infection sites involving virtually every tissue of the body. In general, three types of disease are usually associated with infection by *S. aureus*. These are characterized as superficial or cutaneous infections such as pimples, boils, and toxic epidermal necrolysis; systemic infections such as heart valve disease, bacteremia, and osteomyelitis; and toxinoses such as food poisoning and toxic shock syndrome.

Immunity

Immunity to staphylococcal infections is poorly understood. Normal healthy humans have a high degree of innate resistance to invasive infections. Experimental infections are difficult to establish in animals and require large inocula containing millions of organisms. In humans, the organism is able to colonize mucosal and epidermal surfaces with little resistance, and as long as they remain intact, these barriers are the main source of natural immunity to infection. After invasion, however, phagocytosis by polymorphonuclear leukocytes is the main humoral defense. Because of repeated exposure to *S. aureus* and *S. epidermidis* in natural settings, antibodies to various components of the cell and its products (both cell surface and soluble) are prevalent in animals. Nevertheless, with the exception of toxic shock syndrome where antibody is an important factor in immunity, serological studies have not successfully related immunity and antibody titer. Moreover, prior infection fails to elicit immunity to reinfection. In spite of these drawbacks, vaccine research is being pursued strongly. Although there is currently no vaccine that stimulates active immunity in humans, a vaccine based on fibronectin-binding protein has been shown to confer protective immunity against mastitis in cattle. Among the vaccines being studied for use in humans, the most promising is a polysaccharide conjugate vaccine that was given Fast Track Status by the FDA in 2004 for the prevention of bacteremia in certain at-risk patient populations; however, the vaccine failed to reduce the incidence of *S. aureus* infections when used in clinical trials.

Identification

The clinical laboratory must be able to identify *S. aureus* quickly and accurately. Many types of clinical samples have been utilized for detection. Samples that are heavily contaminated with other bacterial flora (i.e., nasal, skin, or wound specimens) can be grown on solid media or liquid media containing various selective agents that exploit the resistance of staphylococci to NaCl, chemicals such as potassium tellurite and lithium chloride, or antibiotics. Suspect colonies from the primary isolation are then subjected to antibiotic sensitivity determination to provide treatment alternatives. As mentioned above, coagulase determination, an important consideration for treatment decisions regarding CoNS strains, is usually carried out. A variety of other phenotypic markers can be tested if further characterization is desired. These include α- and β-hemolysins, nuclease, proteases, lipase, protein A, and the determination of specific toxins.

Treatment

More than 95% of patients with *S. aureus* infections worldwide do not respond to first-line antibiotics such as penicillin and ampicillin. These strains have been routinely treated with methicillin, but resistance appears in a large number of infections (MRSA). Vancomycin is the most effective treatment for multiresistant strains, but the recent emergence of vancomycin resistance in staphylococci poses a critical problem to effective treatment of these infections. The emergence of strains that are resistant to multiple antibiotics, especially the recent appearance of vancomycin resistance, has spurred renewed interest in antibiotic discovery and vaccine therapy. New drug targets are being investigated to devise novel families of antibiotics. Methicillin resistance is not limited to *S. aureus* and all of the other staphylococcal species that have been sequenced to date are isolates that carry the genes conferring resistance to this antibiotic and some contain plasmids that confer resistance to other compounds used for treatment.

Antibiotic Resistance and MRSA

Nosocomial infections with MRSA have been identified as a problem since the 1960s, with ~20% of bloodstream infections being due to *S. aureus*. Recent data showed that as many as 64.4% of hospital onset *S. aureus* infections reported in US intensive care units were due to MRSA. These types of infections are not only important due to the danger they pose to the patient but they also result in longer hospital stays, higher mortality, and increased costs for alternative treatments. Until recently, the focus of MRSA infections was limited to those that were nosocomial (hospital onset) in nature, with little or no surveillance activity for community-acquired MRSA (cMRSA). This was due in large part to a much lower incidence of cMRSA in relation to hospital-acquired infections. However, in a recent study carried out by the US Centers for Disease Control (CDC) to estimate the burden of invasive MRSA infections in the United States in 2005, cMRSA was identified as a major source of infection. Of the almost 8987 cases of invasive MRSA that were reported, ~85% were related to recent hospitalization or were of hospital onset and 13.7% were classified as cMRSA. The study also showed that infection rates were highest in older patients (>= 65 years), African Americans, and males. Interestingly, the group at lowest risk included young people ranging from 5 to 17 years of age. The study concluded that invasive MRSA affects certain populations disproportionately and that it is a major public health problem primarily related to health care. The researchers also stress the point that MRSA should no longer be confined to healthcare institutions, and that the incidence of cMRSA will most likely continue to increase.

There are multiple reasons that the staphylococci, especially *S. aureus*, have become antibiotic-resistant including overuse of antibiotics in humans, the presence of antibiotics in food and water supplies, and mutation and/or exchange of genes within the genus. Unnecessary prescriptions for antibiotics is one of the main sources contributing to staphylococcal (and other microorganisms) developing resistance. Decades of excessive antibiotic use for colds, flu, and other viral infections that do not respond to these drugs results in low-level exposure to these compounds by normal bacterial flora found in the host. This repeated exposure to sublethal concentrations results in the elimination of the majority of the resident bacteria and selects for spontaneously occurring mutants that are resistant to the antibiotic. Over time, the presence of the antibiotic has no effect on the organism and therefore they are resistant to killing by the drug when it is prescribed for actual treatment. Prescription drugs are not the only source of antibiotics. In the United States, antibiotics can be found in animal feeds especially for beef cattle, pigs, and chickens. The same antibiotics then find their way into municipal water systems when the runoff from feedlots contaminates streams and groundwater. However, antibiotics given in the proper doses to sick animals do not appear to produce resistant bacteria. Even appropriate antibiotic use can contribute to the increase in drug-resistant bacteria because they may not destroy every organism within the population. Bacteria evolve rapidly; so those that survive treatment with one antibiotic are soon capable of resisting others. Bacteria mutate much more quickly than new drugs can be produced, which makes it possible for a given organism to become resistant to all available treatment options. In addition to mutation of genes, the staphylococci are adept at gene transfer via mobile genetic elements such as plasmids, phage, and transposons. By carrying and transferring antibiotic-resistant genes via these mechanisms, antibiotic resistance has become rampant within this group. Although there has not yet been a strain of *S. aureus* identified that is resistant to all of the available antibiotics, there are many that are resistant to the majority of drugs currently available. In fact, it is relatively uncommon for any given clinical isolate to be resistant to only one drug and almost all that are isolated are resistant to penicillin, which was the original drug used to treat most bacterial infections. More importantly, there are now multiple strains that have acquired resistance to what used to be the drug of last resort (vancomycin). Using vancomycin and other more aggressive forms of treatment contribute to the economic burden endured by the patient and hospital, and these types of treatments are also more physically demanding for the patient. For example, vancomycin is generally given in multiple intravenous doses over several weeks, which requires additional specialized care and a longer stay within the hospital. The drug itself often makes the individual feel even worse than before treatment began, with potential side effects including, but not limited to, kidney failure, temporary or permanent hearing loss, neutropenia, anaphylaxis, pain and inflammation at the injection site, severe stomach pain, diarrhea, and fever or chills.

Virulence Factors

Staphylococci produce a wide array of extracellular and cell surface proteins with a large number of these encoded on plasmids and other accessory elements. Because of this, different strains have been shown to exhibit a variable array of toxins, enzymes, and other factors. A list of some of these exoproteins is presented in **Table 3**. The extracellular proteins are subdivided into cell surface-oriented proteins and soluble proteins. All the exoproteins are translated as precursor proteins with signal peptides, which are removed at secretion. In addition, the cell surface proteins also possess a characteristic amino sequence motif (LPXTG) at the C-terminus, which precedes the membrane spanning region and serves as an anchor, linking the protein to the cell wall peptidoglycan. Both types of exoproteins are secreted by Type II secretory mechanisms involving the SecYEG pathway. With the exception of certain bacteriocins, there is no evidence to date that extracellular proteins are secreted by other secretory pathways.

Table 3 Extracellular proteins of *Staphylococcus aureus*

Protein	*Gene locus*
Hemolysins	
• Alpha toxin	Chromosome
• Beta toxin[a]	Chromosome
• Gamma toxin	Chromosome
• Delta toxin[a]	Chromosome
• Panton-Valentine leukocidin	Chromosome/pathogenicity island
Enterotoxins	
• SEA	Bacteriophage/chromosome
• SEB	Chromosome/pathogenicity island
• SEC	Plasmid
• SED	Plasmid
• SEE	Chromosome
• SEG	Chromosome
• SEI	Chromosome
Enzymes and other toxins	
Lipase[a]	Chromosome
Nuclease[a]	Chromosome
V8 Protease[a]	Chromosome
Esterase	Chromosome
Coagulase	Chromosome
Cell wall hydrolase	Chromosome
Hyaluronadase	Chromosome
Staphylokinase	Bacteriophage/chromosome
Protein A	Chromosome
Serine proteases[a]	Chromosome/genomic island
Zinc metalloproteinase aureolysin[a]	Chromosome
Phospholipase C	Chromosome
Leukotoxins	Chromosome/genomic island
Leukocidins	Chromosome/pathogenecity island
Exfoliative toxin A	Chromosome
Exfoliative toxin B	Plasmid
Toxic shock syndrome toxin-1	Chromosome/pathogenicity island
MSCRAAMs	
Clumping factors	Chromosome
Fibronectin-binding proteins A/B	Chromosome
Fibrinogen-binding protein	Chromosome
Collagen-binding protein	Chromosome
Elastin binding protein[a]	Chromosome
Extracellular matrix-binding proteins[a]	Chromosome
Intercellular adhesion proteins[a]	Chromosome

[a]Denotes virulence factors that are also present in *S. epidermidis*.

Soluble Exoproteins

These include a wide array of toxins and enzymes such as food poisoning enterotoxins, exfoliative toxins, toxic shock syndrome toxin, hemolysins, coagulase proteases, lipases, and other enzymes. The exfoliative toxins are proteolytic and attack the epidermis of susceptible animals. Exfoliative toxins cause blistering skin diseases known as bullous impetigo and staphylococcal scalded-skin syndrome (SSSS). Three isoforms of exfoliative toxins (ETA, ETB, and ETD) have been identified in virulent strains of *S. aureus* and four isoforms have been shown to exist in the animal pathogen *S. hyicus*. Clinical manifestation of SSSS is typically seen in neonates and has been termed scalded skin syndrome due to the symptoms that occur culminating in areas of raw, red skin that resembles that of a first-degree burn. The enterotoxins have been shown to act at the interface between the stratum granulosum and stratum spinosum of the epidermis, thereby resulting in characteristic exfoliation of the skin. In other words, there is a loss of keratinocyte cell–cell adhesion in the epidermis. It was initially difficult to determine the mechanism of action for these toxins because purified forms of both ETA and ETB showed no direct protease activity toward multiple targets. However, recent studies have shown that the three isoforms of exfoliative toxins, ETA, ETB, and ETD, are glutamate-specific serine proteases and that ETA and ETB specifically cleave a protein in the epidermal interface called desmogelin 1. ETD has been shown to be encoded within a pathogenecity island and to play a slightly different role in the development of bullous impetigo and scalded skin syndrome disease.

The hemolysins are membrane-damaging proteins whose activity is mediated through pore formation or lipolytic action. The most studied of the hemolysins is α-toxin, which is toxic to a wide range of mammalian cells and is highly hemolytic for rabbit erythrocytes. It is also dermonecrotic and neurotoxic and is produced by almost all strains of *S. aureus*. β-toxin is made in high concentrations, particularly by animal strains of *S. aureus*, and is highly hemolytic for sheep erythrocytes but not rabbit red blood cells. This toxin exhibits phosphorylase C activity that requires magnesium but it has a limited range of activity due to specificity for sphingomyelin and lysophosphatidyl choline. The role of β-toxin in disease is not well understood but the fact that it is made at much higher levels in animal strains suggests that it may provide an advantage in animal hosts as opposed to humans. Another example of an *S. aureus* pore-forming toxin is the Panton–Valentine leukocidin (PVL), which is a cytotoxin that causes leukocyte destruction and tissue necrosis. PVL is encoded on a bacteriophage and has been associated with both staphylococcal skin and pulmonary infections. PVL-containing strains have also been isolated at a higher frequency from patients with severe cMRSA pneumonia and the presence of the toxin is much higher in strains that carry the SCCmecIV cassette than in strains that do not.

Another group of toxins that play a significant role in staphylococcal disease are the enterotoxins. The enterotoxins are responsible for the clinical manifestations of staphylococcal food poisoning and a septic shock-like illness. There are five major classical types of staphylococcal enterotoxins (SEs), and several new SEs or SE-like toxins have recently been identified. Ingestion of these toxins leads to severe gastroenteritis with emesis, nausea, and diarrhea. In addition, the SEs are resistant to extreme heat and are stable over a wide pH range besides being resistant to degradation by a variety of proteases. They are also classified as superantigens, a characteristic that is described in more detail below.

Several of the soluble exoproteins including enterotoxins, exfoliative toxins, and toxic shock toxin are superantigens due to their ability to stimulate mitogenic activity and cytokine production for a wide array of T-lymphocyte haplotypes. They are able to activate specific sets of T-lymphocytes by binding to major histocompatibility complex class II (MHC II) proteins. They bind to the variable region of the T-cell receptor β-chain. The activated cells proliferate and release cytokines/lymphokines, interferon-γ (IFN-γ), and interleukins. Because they exhibit this broad-based activity, they have been called superantigens. This activity is suspected to enhance virulence by suppressing the hosts response to staphylococcal antigens produced during infection. In addition to the factors discussed above, there are many staphylococcal enzymes such as lipase, nuclease, and proteases, and all are presumed to enhance invasiveness through tissue destruction.

Cell Surface Proteins

A major class of cell surface proteins are adhesins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These molecules comprise the main adhesins of the organism and include collagen-binding protein, fibronectin-binding proteins, fibrinogen-binding protein, elastin-binding protein, clumping factor, and the matrix adhesin factor. There may be as many as 12 other surface proteins that contain membrane anchor domains and potentially qualify as MSCRAMMs. A second group of cell surface proteins includes nuclease and protein A. Staphylococcal nuclease is a thermally stable endonuclease able to withstand boiling for 30 min without significant loss of activity. Protein A is able to bind to the nonantigenic F_c fragment of immunoglobulin G, causing the complex to precipitate. Its role in virulence is believed to be in escape from immune surveillance.

Regulatory Mechanisms

The expression of extracellular proteins is largely under the influence of a master genetic circuit called *agr* (accessory gene regulator). This signaling arm of the operon (AgrBDCA) is activated by a quorum-sensing mechanism that depends upon the accumulation of an activating octomeric peptide (processed from the AgrD precursor by AgrB). The peptide triggers increased expression of the entire operon via an integral signal transduction pathway (AgrC, AgrA), upregulating production of the octomer and activating a second promoter that produces an unique regulatory molecule, RNAIII. RNAIII is the effector molecule for regulated protein expression. It is neither translated nor does it bind to the promoter regions of regulated genes. It presumably interacts with other genes, in an unknown way, as both a positive and negative regulator of exoprotein gene expression. RNAIII is required for the expression of soluble exoproteins and represses the expression of cell surface proteins. Because a threshold level of octapeptide is required for activation, RNAIII is not expressed until late in growth. Therefore, cell surface proteins are produced early, presumably to allow the organism to attach and colonize. The RNAIII-induced activation of soluble proteins genes results in a necrotic effect, allowing the organism to invade deeper tissues and become bacteremic. A second locus, *sarA*, modulates expression of the *agr* locus by binding to the promoter region of AgrBDCA. The *sarA* locus is transcribed from three different promoters (sarP1, P2, and P3) that are active at different times during growth. The major regulatory molecule encoded by *sarA* is a 14.5 kDa protein that has been shown to bind to the promoter regions for fibronectin-binding protein A, the collagen adhesion, protein A, and *agr*. It is reported that the *sarA* locus plays a role in transcription of over 100 different gene targets either by direct binding of upstream promoter regions or indirectly due to its effects on other regulatory loci including *agr*. Several *sar* homologues have been identified including SarR, SarS, SarU, and SarY that play a role in regulation of SarA and other factors. With the continued efforts aimed at whole-genome sequencing of staphylococcal isolates, many other regulatory systems that are not discussed in detail here have been identified and characterized. These include Rot, SaeRS, SrrAB, ArlRS, and LytRS.

Another important level of regulation of virulence is regulation of gene expression in response to environmental factors. It has been demonstrated that depending on the environment that the staphylococci encounter they can adapt by altering gene expression for a variety of systems. This is not unusual for bacteria and in fact the main environmental response system that has been described for *S. aureus* and *S. epidermidis* is the sigma B pathway. Sigma factors are bacterial proteins that enable specific binding of RNA polymerase to promoter regions within the DNA. The staphylococcal sigma B response is similar to that described for other Gram-positive pathogens such as *B. subtilis*. Sigma B is an alternative sigma factor that is activated under environmentally stressful conditions such as high salt levels, presence of ethanol, energy depletion, and low pH. Comprehensive studies in *S. aureus* have shown that sigma B regulates gene expression of some factors by directly binding to a specific site upstream of promoters and also by indirectly affecting upstream factors to gene expression. Some of the virulence factors that have been shown to be affected due to sigma B expression include coagulase, fibronectin-binding protein B, biofilm formation, and α-toxin. A change in resistance levels to some antibiotics that affect the bacterial cell wall has also contributed to overexpression of sigma B. It is important to note that some strains of *S. aureus* have a mutation in the sigma B activator (RsbU) that renders them sigma B defective. These strains are also capable of growing under environmentally stressful conditions, which suggests that there are additional systems that have yet to be identified and that must play a role in virulence factor expression.

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Strain Improvement

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Defining Statement
Introduction
Attributes of Improved Strains
Significance, Impact, and Benefits
Strategies for Strain Development
Improved Strain Performance Through Engineering Optimization
Further Reading

Glossary

DNA recombination A laboratory method in which DNA segments from different sources are combined into a single unit and manipulated to create a new sequence of DNA.

fermentation A metabolic process whereby microbes gain energy from the breakdown and assimilation of organic and inorganic nutrients.

gene Physical unit of heredity. Structural genes, which make up the majority, consist of DNA segments that determine the sequence of amino acids in specific polypeptides. Other kinds of genes exist. Regulatory genes code for synthesis of proteins that control expression of the structural genes, turning them off and on according to circumstances within the microbe.

gene cloning Procedure employed where specific segments of DNA (genes) are isolated and replicated in another organism.

genetic code The linear sequence of the DNA bases (adenine, thymine, guanine, and cytosine) that ultimately determines the sequence of amino acids in proteins. The genetic code is first ‘transcribed’ into complementary base sequences in the messenger RNA molecule, which in turn is ‘translated’ by the ribosomes during protein biosynthesis.

genetic recombination When two different DNA molecule are paired, those regions having homologous nucleotide sequences can exchange genetic information by a process of natural crossover to generate a new DNA molecule with a new nucleotide sequence.

interspecific protoplast fusion Method for recombining genetic information from closely related but nonmating cultures by removing the walls from the cells.

metabolic engineering A scientific discipline that integrates the principles of biochemistry, chemical engineering, and physiology to enhance the activity of a particular metabolic pathway.

mutation Genetic lesion or aberration in DNA sequence that results in permanent inheritable changes in the organism. The strains that acquire these alterations are called mutant strains.

plasmid An autonomous DNA molecule capable of replicating itself independently from the rest of the genetic information.

primary metabolites Simple molecules and precursor compounds such as amino acids and organic acids that are involved in pathways that are essential for life processes and the reproduction of cells.

secondary metabolites Complex molecules derived from primary metabolites and assembled in a coordinated fashion. Secondary metabolites are usually not essential for the organism’s growth.

Abbreviations

EMS	ethyl methanesulfonate
HPLC	high-pressure liquid chromatography
MSG	monosodium glutamate
NIPAB	6-nitro-3-phenylacetamidobenzoic acid
NTG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
ONPG	2-nitrophenyl-β-D-galactopyranoside
PNPP	4-nitrophenylphosphate

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Defining Statement

The science and technology of designing, breeding, manipulating, and continuously improving the performance of microbial strains for biotechnological applications is referred to as 'strain improvement'. The science behind developing improved cultures has been enhanced recently by a greater understanding of microbial biochemistry and physiology, coupled with advances in fermentation reactor technology and genetic engineering. In addition, the availability and application of user-friendly analytical equipment such as high-pressure liquid chromatography (HPLC) and mass spectroscopy, which raised the detection limits of metabolites, have also played a critical role in screening improved strains.

Introduction

The use of microbes for industrial processes is not new. Improving the commercial and technical capability of microbial strains has been practiced for centuries through selective breeding of microbes. In making specialty foods and fermented beverages (such as alcohol, sake, beer, wine, vinegar, bread, tofu, yogurt, and cheese), specific strains of bacteria and fungi isolated by chance have been employed to obtain desirable and palatable characteristics. Now, with integrated knowledge of biochemistry, chemical engineering, and physiology, microbiologists have taken a more scientific approach to the identification of microbial strains with desired traits.

Later spectacular successes observed in improvement of the industrial strains by mutation and genetic manipulations in the production of penicillin and other antibiotics led to strain development as a driving force in the manufacture of pharmaceuticals and biochemicals. Microbes are now routinely used in large-scale processes for the production of lactic acid, ethanol fuel, acetone–butanol, and riboflavin as well as for the commercial production of enzymes such as amylases, proteases, and invertase. Efforts were also made by chemical engineers to improve fermenter designs on the basis of understanding the importance of culture media components, sterile operations, aeration, and agitation. Today, production of hormones, steroids, vaccines, monoclonal antibodies, amino acids, and of antibiotics are testimonies to the important role of strain improvement in the pharmaceutical industry.

The arm of this article is to briefly describe strategies employed in strain improvement, the practical aspects of screening procedures, and the overall impact that strain improvement has on the economics of fermentation processes. Readers are also urged to review the additional articles listed in 'Further Reading', especially the basic concepts as well as the theoretical basis of genetic mutations and screening improved strains.

Attributes of Improved Strains

Microbial strain improvement cannot be defined simply in terms of modifying the strain for overproduction of bioactive compounds. Strain improvement should also be viewed as making the fermentation process more cost-effective. Some of the traits unique to fermentation process that make a strain 'improved' are the ability to (1) assimilate inexpensive and complex raw materials efficiently; (2) alter product ratios and eliminate impurities or by-products problematic in downstream processing; (3) reduce demand on utilities during fermentation (air, cooling water, or power draws); (4) excrete the product to facilitate product recovery; (5) provide cellular morphology in a form suitable for product separation; (6) create tolerance to high product concentrations; (7) shorten fermentation times; and (8) overproduce natural products or bioactive molecules not synthesized naturally, for example, insulin.

Need for Strain Improvement

Microbes (fungi, bacteria, actinomyces) that live freely in soil or water and produce novel compounds of commercial interest, when isolated from their natural surroundings, are not ideal for industrial use. In general, wild strains cannot make the product of commercial interest at high enough yields to be economically viable. In nature, metabolism is carefully controlled to avoid wasteful expenditure of energy and the accumulation of intermediates and enzymes needed for their biosynthesis. This tight metabolic and genetic regulation, and synthesis of biologically active compounds, is ultimately controlled by the sequence of genes in the DNA that program the biological activity. To improve microbial strains, the sequence of these genes in the DNA that program the biological activity. To improve microbial strains, the sequence of these genes must be altered and manipulated. In essence, microbial strain improvement requires alteration and reprogramming of the DNA (or the genes) in a desired fashion to shift or bypass the regulatory controls and check-points. Such DNA alterations enable the microbe to devote its metabolic machinery to producing the key biosynthetic enzymes and increasing product yields. In some cases, simple alteration in DNA can also lead to structural changes in a specific enzyme that increases its ability to bind to the substrate, enhance its catabolic activity, or make itself less sensitive to the inhibitory effects of a metabolite. On the contrary, when the changes are made in the regulatory region of the gene (such as the promoter site), it can lead to deregulation of gene expression and overproduction of the metabolite. A typical example is overproduction of the enzyme amylase, where specific constitutive mutants have been developed that produce the enzyme even in the absence of the starch inducer.

Knowledge of the functions of enzymes, rate-limiting steps in pathways, and environmental factors controlling synthesis further helps in designing screening strategies. The outcome of the strain selection, however, depends primarily on the kind of improvement desired from the microbe. For instance, increased product yield that involves the activity of one or more genes, such as enzyme production, may be enhanced simply by increasing the gene dosage. Molecules such as secondary metabolites and antibiotics that are complex in structure and require a coordinated as well as highly regulated biosynthetic process, however, may require a variety of alterations in the genome to derive a high yielding strain. Apart from modifying the strains genetically, the success of a strain improvement program also depends on developing and combining more efficient ways of screening, testing, and confirming the improved and high yielding status of the mutants against a background of nonimproved strains.

Significance, Impact, and Benefits

Strain improvement is the cornerstone of any commercial fermentation process. In most cases, it determines the overall economics. Fermentation economics is predominantly determined by manufacturing cost per unit of product made (e.g., 'dollars per pound') and the cost associated with plant construction and start-up. Although lower fermentation manufacturing and capital costs can be anticipated from fermenter engineering design, improvement of microbial strains offers the greatest opportunity for cost reduction. Great efforts are therefore expended to develop industrial strains that have an increased ability to produce the compound of interest at a faster rate. Enhanced productivity of the fermentation process through strain improvement (more product/vessel/unit time, e.g., grams per liter per hour) is one factor that makes the most impact. It can determine the ability of a manufacturing process to meet additional demands without adding more fermenters. Furthermore, the strain that can synthesize a higher proportion of the product using the same amount of raw material can also reduce material and manufacturing costs significantly. For example, strains that utilize low-cost materials such as starch or corn syrup, or spent products like molasses (instead of refined glucose), can reduce fermentation costs significantly.

Improvement of industrial strains is clearly justified when one takes into account the additional anticipated capacity and extra fermenters (capital cost) required to meet the throughput in the absence of titer gains and strain development efforts. Through strain improvement, one can free up fermenters and facilitate the launching of other fermentation products in the pipeline. Also of great importance is the use of genetically engineered microbes that manufacture nonmicrobial products such as insulin, interferon, human growth hormone, and viral vaccines that cannot be produced efficiently by other manufacturing processes.

Strategies for Strain Development

Several procedures are employed to improve microbial strains. All bring about changes in DNA sequence. These changes are achieved by mutation, genetic recombination, or the modern DNA splicing techniques of 'genetic engineering'. Each procedure has distinct advantages. In some cases, a combination of one or more techniques is employed to attain maximum strain improvement.

Mutation

Microbes, generation after generation, generally inherit characteristics identical to their parents. However, when changes occur in the DNA, they too are passed on to daughter cells and inherited in future generations. This permanent alteration of one or more nucleotides at a specific site along the DNA strand is called a genetic mutation. The strain that harbors the mutation is called a mutant strain. Although a gene consists of hundreds or even thousands of base pairs, a change in the just one of these bases can have a significant change in the function, operation, and expression of the gene or in the function of its protein product. A mutant, by definition, will differ from its parent strain in genotype, the precise nucleotide sequence in the DNA of the genome. In addition, the visible property of the mutant, its phenotype, may also be altered relative to the parent strain.

A point mutation may be associated with a change in a single nucleotide, through substitution of one purine for another purine or substitution of one pyrimidine for another pyrimidine (transition), or through substitution of one purine by a pyrimidine or vice versa (transversion). Mutations may also result from deletion of one or more base pairs, insertion of base pairs, or rearrangement of the chromosome due to breakage and faulty reunion of the DNA. These changes in base pair arrangements can alter the 'reading frame' of the gene (frameshift mutations), and during the transcription and translation process also change the amino acid sequence in the resulting protein. Most mutations occur on a chromosome structure at a specific site or locus (gene mutations).

Genetic mutations do occur spontaneously, at low frequency at any point along the gene (10^{-5}–10^{-10} per generation). Some mutations are the result of integration or excision of insertion sequence elements and result in subtle modification of the genetic sequence. In many cases mutations are harmful, but certain mutations occur that make the organism better adapted to its environment and improve its performance. The potential for a microbe to mutate is an important property of DNA since it creates new variation in the gene pool.

Modification of the strain through mutation can also be induced at will, by subjecting the genetic material to reaction with a variety of physical and chemical agents called mutagens. Examples of some known mutagenic agents are listed in **Table 1**. Each agent includes DNA alterations in a specific manner, and in some cases, an agent may induce more than one type of lesion. Most cause some damage to the DNA through deletion, addition, transversion, or substitution of bases or breakage of DNA strands (**Table 1**). Although microbes have systems to repair the damaged of altered DNA and return it to its original form, the repairing and editing mechanisms are not errorproof. Thus, when DNA reacts with mutagenic agents for longer periods of time, the damage in the DNA cannot be repaired to the correct genetic sequence with the same rapidity and accuracy as in normal circumstances. The microbes (progeny) that survive the changes in their genetic DNA sequence usually acquire an altered genetic code for 'reprogrammed' metabolic and biosynthetic activity. The frequency of bacterial mutation for a particular trait is low: 1 per 10^4–10^{10} cells per generation.

In addition to mutation, alteration in DNA can also occur by genetic recombination. Here information from two similar but different DNA molecules is brought together and recombined by crossing-over, resulting in novel DNAs of different lengths so that new combinations of mutations are produced. This allows the circumvention of slow leaps to obtain new combinations of desired characteristics in microbes. Genetic engineering is usually employed to create targeted mutations on the genes, unlike other methods of mutation that are random. It must be emphasized that this technology is not a way of constructing new forms of life. Even the genetic materials of the simplest organisms are highly complex, and insertion of a few genes from an unrelated organism will not create a new microbe.

On the basis of the method of screening and selection chosen, there are basically two methods of improving microbial strains through random mutation: (1) random selection and (2) rationalized selection.

Random selection

Random mutagenesis and selection is also referred to as the classic approach or nonrecombinant strain improvement procedure. Improved mutants are normally identified by screening a large population of mutated organisms, since the mutant phenotype may not be easy to recognize against a large background. After inducing mutations in the culture, the survivors from the population are randomly picked and

Table 1 Mutagens employed for strain development

Mutagen	*Mutation induced*	*Impact on DNA*	*Relative effect*
Radiation			
Ionizing radiation			
1. X-rays, gamma rays	Single- or double-strand breakage of DNA	Deletions, structural changes	High
Short wavelengths			
2. Ultraviolet rays	Pyrimidine dimerization and cross-links in DNA	Transversion, deletion, frameshift, GC → AT transitions	Medium
Chemicals			
Base analogues			
3. 5-Chlorouracil, 5-bromouracil	Faulty base pairing	AT → GC, GC → AT transition	Low
4. 2-Aminopurine	Errors in DNA replication		Low
Deaminating agents			
5. Hydroxylamine (NH_2OH)	Deamination of cytosine	GC → AT transition	Low
6. Nitrous acid (HNO_2)	Deamination of A, C, and G	Bidirectional translation, deletion, AT → GC, and/or GC → AT transition	Medium
Alkylating agents			
7. NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine)	Methylation, high pH	GC → AT transition	High
8. EMS (ethyl methanesulfonate)	Alkylation of C and A	GC → AT transition	High
9. Mustards, di-(2-chloro-ethyl) sulfide	Alkylation of C and A	GC → AT transition	High
Intercalating agents			
10. Ethidium bromide, acridine dyes	Intercalation between two base pairs	Frameshift, loss of plasmids, microdeletions	Low
Biological			
11. Phage, plasmid, DNA transposons	Base substitution, breakage	Deletion, duplication, insertion	High

tested for their ability to produce the metabolite of interest. This approach has the advantage of being simple and reliable. Moreover, it offers a significant advantage over the genetic engineering route alone by yielding gains with minimal start-up and sustaining such gains over years despite a lack of scientific knowledge of the biosynthetic pathway, physiology, or genetics of the producing microbe. This empirical approach has been widely adapted by the fermentation industry, following the successful improvement in penicillin titers since World War II. One drawback to the random selection approach is that it relies on nontargeted, nonspecific gene mutations, so many strains need to be screened to isolate the improved mutant in the mixed population. In addition, when the culture is mutagenized, multiple mutations may be introduced in the strain. This may result in the enfeeblement of the organism lacking the properties of interest.

This process of strain improvement involves repeated applications of three basic principles: (1) mutagenesis of the population to induce genetic variability, (2) random selection and screening from the surviving population of improved strains by small-scale model fermentation, and (3) assaying of fermentation broth/agar for products and scoring for improved strains. It must be emphasized that the action of the mutagenic agent on DNA not only can cause genetic alteration but can also induce cell death, owing to irreversible damage to the DNA or formation of lethal mutations. Hence, after the mutagenic treatment, mutants are sought among the surviving population with the anticipation that each of the surviving cells harbors one or several mutations. Each time an improved strain is derived through mutation, it is used again as the parent strain in a new cycle of mutation, screening by fermentation (liquid or solid), and assay (**Figure 1**). This random procedure of mutant selection is continued until a strain is derived that is statistically superior in performance to the control strain prior to the mutagenic treatment. The objective of mutagenesis is to maximize the frequency of desired mutations in a population while minimizing the lethality of the treatment. For this purpose, nitrosoguanidine (NTG) has been the mutagen of choice because it offers the highest possible frequency of mutants per survivor. The efficiency of the random selection process is dependent on several factors: the type of culture used (such as spores or conidia), mutagen dose and exposure time, the type and damage to DNA, conditions of treatment and posttreatment, frequency of mutagen treatment, and the extent of detectable yield increase.

In addition to the mutation conditions, the test or quantitative and analytical screening procedures employed (bioassays, radioimmunoassays, chromatography, HPLC)

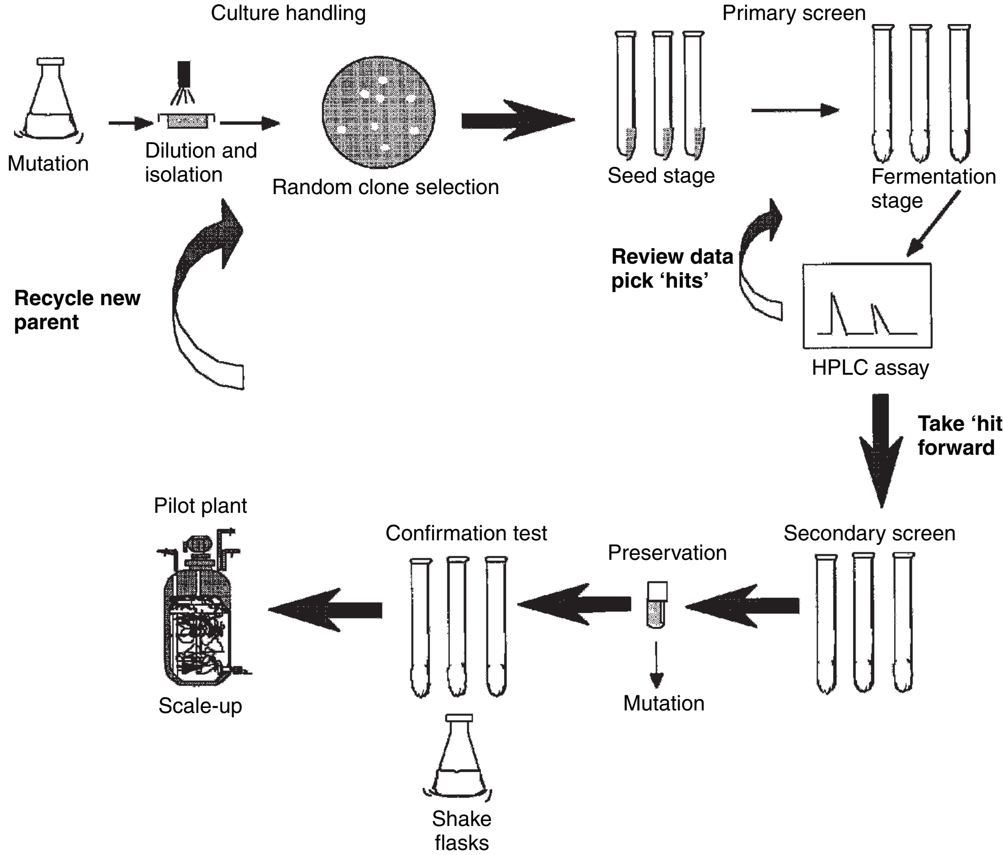

Figure 1 Typical steps in mutation and random strain selection process.

also play a critical role in successful isolation of superior mutants. The ability to detect a gain mutant among the randomly selected mutants is greatly influenced by the process as well as the variability within the process and the actual titer differences between the improved strain and the control. The screening procedure therefore is usually designed to maximize the precision and selectivity of improved cultures (gain per sample tested) and to minimize the variability (measured as the coefficient of variation) when treating the unmutagenized control and reference samples. All the strains tested, including the control, are normally worked up all the way head-to-head from the initial cell clone stage to the final screening stage. This is essentially the test of significance. As such, if all the treatment conditions were the same, a successful test should show a statistical difference between the means of the control and improved cultures.

Putative mutants isolated after a primary run are subjected to secondary and tertiary confirmations (replications and repetitions) to raise the level of confidence and observe the anticipated titer differences on data collections (**Figure 1**). A desired improvement in the strain is typically obtained with less testing if the selection system is less susceptible to variability and if the coefficient of variation is lower. Considerable efforts are therefore directed at troubleshooting important handling procedures to identify and eliminate key contributors to the errors in the process. Furthermore, the screening strategy is carefully chosen so that the medium and fermentation parameters mimic large-scale production. This increases the possibility that the improved performance of the mutant will be achieved at scale-up. The random approach of strain selection relies on delivering small incremental improvements in culture performance. Although the procedure is repetitive and labor intensive, this empirical approach has a long history of success and has given dramatic increases in titer improvement, as best exemplified by the improvements achieved for penicillin production in which titers over $50\,\mathrm{g\,l^{-1}}$ are reported – a 4000-fold improvement over the original parent strain. W. Crueger and A. Crueger have also cited certain actinomyces or fungal strains capable of overproducing metabolites in quantities as high as $80\,\mathrm{g\,l^{-1}}$. Not surprisingly, therefore, pharmaceutical and other fermentation industries typically adopt this technique for selection of improved mutants for many of their processes. The historic successes (e.g., production of antibiotics and other secondary metabolites, enzymes, and amino acids) bear testimony to creating superior strains through this procedure.

The procedure of mutation followed by random selection is laborious and requires screening a large number of strains to obtain desired mutants. This is because in random screening procedures a high percentage of mutants examined will be carried over as survivors from the mutagenesis and will exhibit the same or lower yields than the parent strain. Factors that impact the success of the random program and accelerate strain improvement are the following: the extent of yield improvement, the frequency of induced mutations, the amount of time for turn-around of the mutation selection cycle, and the testing capacity. In addition, the success of a strain improvement program also depends on resource allocation. The key labor-intensive steps in classic strain improvement programs include the isolation of individual mutated cultures, preparation and distribution of sterile media, transfer of clones and their inoculum to initiate fermentation, assays of fermentation broth, and repeated confirmations. Furthermore, the more complex the regulation and biosynthesis of a desired compound, the greater the number of strains that need to be evaluated and replicated. As a rule, mutants with very high yields are rarer than those with subtle improvements. So, to increase the odds, a larger number of improved strains are examined, raising the probability of detecting improved strains. Thus, if the strain improvement program is operated manually, successful improvements detected will roughly be proportional to the number of personnel allocated. The advantage of manual screening is in the trained eyes of the microbiologists. They can visually detect the alteration in the morphology, pigmentation, and growth characteristics of mutants during the selection process. In fact, isolation of pelleted strains of filamentous organisms is commonly based on morphological criteria.

To increase the efficiency of random selection, ways by which the key steps in the process can drive the throughput higher without adding labor are typically sought. In some instances high-throughput screens have been automated with robotics technology. This allows screening of large populations with minimum resources by miniaturizing wherever possible the equipment required and constructing an automated integrated system. In an industrial system sterile media are robotically dispensed in custom-designed sterilizable and cleanable/disposable modules, each having over 100 tubes or bottles. Individual clones are detected by an optical system and plugged from an agar-based medium into liquid seed medium. The inoculations of seed-stage culture to fermentation vials are also accomplished by robots. The extraction and HPLC analysis of the fermentation broth is also automated to match the throughput of the screening stage. The advantage of such automation is that it facilitates the capture and downloading of process data and allows statistical process control approaches to be implemented where refinement of the process is required. The success of automated programs requires skillful microbiologists, and constant monitoring and evaluation of the screening system to ensure that all aspects of the automation are functioning efficiently without introducing variability. The significant disadvantage of robotic systems is the initial high capital investment and continued maintenance of equipment and software.

Although other sophisticated techniques are being developed to generate improved strains, random high-throughput selection and mutation will continue to be an integral part of any strain improvement program. The random approach is least useful for microbes that are less susceptible to mutagenesis, such as some fungi (owing to their diploid or polyploid genome structure) and bacteria with very efficient repair systems. In a typical manually operated strain improvement project, the expected frequency of gain could be of the order 1 in 10 000, where about 10 000 mutants may pass through the primary screen before a higher producing candidate is identified. However, as the titer increases, depending on the pathway, the organism, the product, and the history of the production strain, significantly larger gains are required to detect an improved mutant. The use of prescreening and rational selection allows for a significant improvement in the efficiency of the selection process.

Rationalized selection

An alternate approach to random screening requires a basic understanding of product formation and the fermentation pathway; this can be acquired through radioisotope feeding studies and isolation of mutants blocked in various pathways. These observations can shed light on the metabolic checkpoints, and suggest ways to isolate specific mutants. For example, environmental conditions (pH, temperature, aeration) can be manipulated, or chemicals can be incorporated in the culture media to select mutants with desired traits. This approach is used in many instances as a prescreen, since selecting for a particular mutant is unlikely to guarantee a hyperyielding mutant. In some instances, by adding toxic substances to the media, the sensitive parent strains are prevented from growing, and only the resistant mutant clones propagate. Such an enrichment procedure has been used to isolate mutants with increased biosynthetic capacity through a change in a regulatory mechanism (leading to either an enzyme resistant to inhibition or an enzyme that is expressed constitutively) or mutants that are modified in the transport or degradation of compounds, which ultimately leads to higher product formation. This rationalized technique is powerful, and when the logic behind the mutant selection criteria is sound, the effectiveness of mutant gains is much greater than with random selection.

Rationalized selection for strain improvement does not generally require a sophisticated understanding of molecular biology to manipulate environmental or cultural conditions. It does, however, require some understanding of cellular metabolism and product synthesis to design the right media or environmental conditions. The procedure is useful in selecting strains overproducing metabolites, antibiotics, simple molecules, amino acids, or enzymes. Some of the mutants derived through rationalized selections are described below.

Auxotrophic mutants

Many metabolic processes have branched pathways, and isolating mutants blocked in one branch of the metabolic pathway can cause accumulation of simple products such as amino acids, nucleotides, and vitamins made by other branches. Auxotrophic strains are blocked at some point in a pathway vital for growth, and unless the specific nutrients or products of the pathway are supplied in the media, the auxotrophs do not survive. Auxotrophs are primarily isolated by plating the mutagenized population on a complete medium that has all the nutrients needed for growth. The clones are then replica-plated to minimal medium lacking some specific nutrients, and auxotrophs that fail to grow on minimal media are identified. Most auxotrophic strains give poor antibiotic yields; however, some prototrophic revertants have demonstrated improved antibiotic production such as in tetracycline production.

Regulatory mutants

Since anabolism and catabolism in any organism are tightly regulated, selection and screening of microbes with less efficient regulation, and optimizing culture conditions, can lead to relaxed regulation and overproduction of microbial products. A broad understanding of metabolic pathway bottlenecks is necessary for a rational approach to developing improved regulatory mutants. Isolating strains relaxed in regulation can usually be accomplished by selecting strains desensitized to feedback inhibition (enzyme activities) or feedback repression (enzyme synthesis) involved in the pathway. One difficulty in applying analogue-resistant mutants to strain improvement is that many analogues of primary metabolities need to be tested and some either do not inhibit growth or inhibit growth only at very high concentrations.

Mutants resistant to feedback inhibition In many microbes, the end products of metabolism, when accumulated in the microbial cell, inhibit the enzyme activities of many pathways. The end product causes conformational changes by binding to a specific (allosteric) site on the enzyme, and inhibits activity. The binding is usually noncompetitive. Mutation in the structural gene, however, can alter the enzyme binding site and prevent these inhibitory effects. By studying the interaction of various analogues of end products and their resistance, improved strains can be selected that lack feedback inhibition and thus overproduce metabolites of interest. For example, some analogues (acting through these regulatory controls) prevent the synthesis of compounds required for growth and thus cause cell death. Supplementing the screening medium with these analogues selects only mutants with altered enzyme structure and desensitized to inhibition effects to grow. Such procedures have led to development of superior mutant strains of *Arthrobacter*, *Bacillus*, *Streptomyces*, *Aspergillus*, and *Corynebacterium* that overproduce amino acids, nucleotides, and vitamins. In some cases, the rational selective agent is biological rather than chemical. Resistance to actinophage has been used to isolate superior vancomycin-producing strains of *Streptomyces orientalis* (Soviet Union Patent 235–244-A, 1969).

Mutants resistant to repression Here intermediates, products of catabolism (derived from breakdown of compounds containing carbon, nitrogen, or phosphorus), or end products regulate the amount of biosynthetic enzymes synthesized and, therefore, the amount of final product formed. However, mutations at the operator site or other regulatory sites on the gene relieve such end-product repression and allow overproduction of the biosynthetic enzyme. For example, it is well known that antibiotics inhibit their own biosynthesis (e.g., penicillin, chloroamphenicol, puromycin, and streptomycin), where key enzymes required for the architecture of these complex molecules are repressed. Mutant strains less sensitive to antibiotic production are therefore isolated to provide higher yields. In a similar context, constitutive mutants have been selected that form enzymes (amylase, glucoamylase, lipase, and protease) independent of cultural conditions or the presence of inducing compounds.

Resistance to an antimetabolite is not the sole means of selecting product-excreting mutants resulting from a desensitized enzyme system. Removing enzymes sensitive to feedback repression or the end product that causes inhibition during fermentation also accelerates productivity. Elimination of end-product inhibition or repression effects have been demonstrated by adding chemicals during the fermentation process to trap the end product or the inhibitor. *In situ* end-product extraction, or adding a mechanical device during fermentation (such as specific membrane modules with a particular molecular weight cutoff), allows the percolation of the final product from accumulating in the broth. Increasing permeability of the cell membrane is another method of controlling intracellular product accumulation, enhancing the extracellular metabolite flux. This approach has been exploited to improve the titers of monosodium glutamate (MSG) from *Corynebacterium*, *Micrococcus*, and *Brevibacterium*.

Last, ways of stabilizing the activity of enzymes involved in the assembly of molecules have been reported to augment product formation and strain performance. For example, in gramicidin biosynthesis amino acids are added to stabilize *in vivo* gramicidin *S* synthetase enzymes and prolong the longevity of biosynthetic activity.

Other procedures

Mutant strains suspected of metabolic impairment can also be assayed visually for the presence or absence of specific enzyme activities by plating and spraying on the 'diagnostic' solid or liquid culture medium with selective reagents, dyes, or an indicator organism. For example, the agar plug method has been used to detect production of an antibiotic by measuring the extent of growth inhibition of an organism sensitive to the antibiotic. The diameter of the resulting zone of inhibition serves as a measure of antibiotic production. Other procedures rely on the use of chromogenic agents, which are normally converted to a visible product by a specific biochemical reaction or reorganization of the redox level in the media. This leads to visual detection from the large background population of a specific strain having the biochemical activity of interest. Examples of these detection substrates are phenol red for acid–base reactions, 2-nitrophenyl-β-D-galactopyranoside (ONPG) for galactosidase, 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) for penicillin amidase, 4-nitrophenylphosphate (PNPP) for phosphatase, nitrocefin for β-lactamase inhibition, and azocasein for proteinases. Tetrazolium and methylene blue (EMB, eosin methylene blue agar for *Escherichia coli* and other coliforms) are commonly employed for detection of oxidation–reduction reaction complexes exhibited by strains of interest. These reactions can be coupled into high-throughout screening systems, giving the possibility of targeting whole cells or isolated enzymes for strain selection. For example, carotenoids have been demonstrated to protect *Phaffia rhodozyma* against singlet oxygen damage. A combination of Rose Bengal and thymol in visible light has been employed to select carotenoid-overproducing strains. Enrichment with a singlet oxygen system led to development of mutants with increases in certain carotenoids but a decrease in astaxanthin.

Genetic Recombination

In addition to the manipulation of microorganisms by mutation, the techniques of genetic recombination can be employed to get new strains containing novel combinations of mutations and superior microbial strains. Generally, genetic recombination methods include those techniques that combine two DNA molecules having similar sequences (homologues). Through the special event of crossing-over, they are reunited to give a new series of nucleotide sequences along the DNA that are stable, expressible genetic traits. This mechanism of gene alteration and strain modification is called genetic recombination. This definition includes the techniques of protoplast fusion, transformation, and conjugation. Most recently, recombinant DNA technology has been employed to assemble new combinations of DNA *in vitro*, which are then reinserted into the genome of the microbe, creating new varieties of microbe not attainable through traditional mutation and rationalized selection approaches. This approach overlaps the other methods to some extent in that it involves tranformation of microbes with laboratory-engineered specific recombinant molecules via plasmid or phage vectors.

Protoplast fusion

Fusing two closely related protoplasts (cells whose walls are removed by enzyme treatment) is a versatile technique that combines the entire genetic material from two cells to generate recombinants with desired traits that cannot be obtained through a single mutation. The technique has the advantage of producing hybrids from cells that are sexually incompatible. The procedure of forced mating allows mingling of DNA that is not dependent on appropriate sex factors and is not influenced by barriers of genetic incompatibility.

The procedure relies on stripping the cell wall of the microbes with lytic enzymes, stabilizing the fragile protoplasts with osmotic stabilizing agents, and using a chemical agent or an electric pulse (electrofusion) to induce membrane fusion and to form a transient hybrid. In the hybrid, the genes align at homologous regions, and crossing-over of genes creates recombination within the fused cells. After recombination, the protoplasts are propagated under specific conditions that favor regeneration of cell walls. The unwanted parents are discriminated against by incorporating selective markers in the screening process (e.g., auxotrophy, extracellular enzymes, morphological differences, and levels of antibiotic produced) so that only recombinants grow and form viable cells. The efficiency of the technique is influenced by the fragility of cells, the types of genetic markers, the fusing agent used, and the protoplast regeneration capability.

The use of protoplast fusion has been reported to improve a wide range of industrial strains of bacteria and fungi including *Streptomyces*, *Nocardia*, *Penicillium*, *Aspergillus*, and *Saccharomyces*. This technique is frequently employed in the brewing industry for improving yield and incorporating traits such as flocculation to aid beer filtering, efficient utilization of starch, contamination control, and minimizing off-flavors during brewing. Many of these traits are not easily achievable through simple mutation. One advantage of protoplast fusion is the high frequencies at which recombinants are produced under nonselective conditions in the absence of sex factors and without need of specific mating types. In *Streptomyces coelicolor* frequencies as high as 20% have been observed. Another interesting feature is that more than two strains can be combined in one fusion. In some instances, four strains have been fused to yield recombinants containing genes derived from all four parents. This approach can be extremely useful in accelerating strain development. However, because of the absence of control over the amount of genetic material from any one strain retained in the recombinant, protoplast fusion may not improve the strain in the desired fashion. The big disadvantage to this approach has been the genetic instability of the fused strains and the lack of control over which genetic alterations occur. A detailed description of protocols and considerations for the application of protoplast fusion to a variety of industrial microbes is available.

Transformation

Transformation is the process involving the direct uptake of purified, exogenously supplied DNA by recipient cells or protoplasts. When this occurs, the donor DNA may either combine with the recipient DNA or exist independently in the cell. This leads to changes in the amount and organization of the recipient microbe DNA, hopefully improving it with some of the characteristics coded by the donor DNA. Transformation can be mediated by total genomic DNA or cloned sequences in plasmid of phage DNA. Essentially, the cultures to be transformed are cultivated in a specific physiological manner to develop the competency to make them readily accept foreign DNA. Having selectable markers on the donor DNA allows easy identification of transformants. This procedure allows the transfer of genetic material between unrelated organisms. Certain microorganisms have a well-established gene cloning system that provides a great potential for improving strains by transformation. Transformation methods for strain improvement pertaining to primary or secondary metabolites have been demonstrated in *Streptomyces*, *Bacillus*, *Saccharomyces*, *Neurospora*, and *Aspergillus*.

Conjugation

Conjugation introduces mutational changes in microbes through unidirectional transfer of genetic material from one strain to the other; it is mediated by plasmid sex factors. Conjugation requires cell-to-cell contact and DNA replication. This mode of genetic exchange can achieve transfer of chromosomal DNA or plasmid DNA. Several strains have been modified by this procedure to make them resistant to specific antibiotics and microbial contamination.

The application of conjugate plasmid recombination technology is employed for strain improvement of *Lactococcus* starter cultures in the dairy industry, which is often plagued by problems with phage. Phage infection can lead to slow acid production, which can economically impact a cheese factory. Furthermore, owing to the non-aseptic nature of the dairy starter culture (open vat in cheese making, the presence of mixed flora in milk), phage-resistant strains are desired as starter cultures for fermentation. Various naturally occuring phage-resistant strains have a number of resistance mechanisms (e.g., abortive infection, restriction/modifications, and absorption inhibitions) that in many instances are carried on conjugative plasmids. Several lactic acid bacteria have therefore been modified by conjugation and transformation procedures to acquire phage resistance in dairy starter cultures. Typical methods for conjugative transfer involve mating by donor and recipient cells on milk agar, followed by harvesting of cells and further isolation on selective medium. This procedure has been applied successfully to construct nisin-producing *Lactococcus* strains.

Cloning and Genetic Engineering

In vitro *recombinant DNA technology*

By employing restriction endonucleases and ligases, investigators can cut and splice DNA at specific sites. Some endonucleases have the ability to cut precisely and generate what are known as 'sticky ends'. When different DNA molecules are cut by the same restriction enzyme, they possess similar sticky ends. Through a form of biological 'cut and paste' processes, the lower part of the one DNA is made to stick well onto the upper part of another DNA. These DNA molecules are later ligated to make hybrid molecules. The ability to cut and paste the DNA molecule is the basis of 'genetic engineering'. A useful aspect of this cut-and-paste process involves the use of plasmid, phage, and other small fragments of DNA (vectors) that are capable of carrying genetic material and inserting it into a host microbe such that the foreign DNA is replicated and expressed in the host. A wide array of techniques can now be combined to isolate, sequence, synthesize, modify, and join fragments of DNA. It is therefore possible to obtain nearly any combination of DNA sequence. The challenges lie in designing sequences that will be functional and useful.

The protocol to modify and improve strains involves the following steps:

1. Isolate the desired gene (DNA fragment) from the donor cells.
2. Isolate the vector (a plasmid or a phage).
3. Cleave the vector, align the donor DNA with the vector, and insert the gene into the vector.
4. Introduce the new plasmid into the host cell by transformation or, if a viral vector is used, by infection.
5. Select the new recombinant strains that express the desired characteristics.

For successful transfer of a plasmid/phage vector, it must contain at least three elements: (1) an origin of replication conferring the ability to replicate in the host cell, (2) a promoter site recognized by the host DNA polymerase, and (3) a functional gene that can serve as a genetic marker. A great deal of literature exists on the theoretical overviews, and laboratory manuals on the use of recombinant DNA for strain modification and improvements are available.

Site-directed mutagenesis for strain improvement

So far the mutations and the modifications of the strains discussed have been randomly directed at the level of the genome of the culture. The application of recombinant technology and the use of synthetic DNA now make it possible to induce specific mutations in specific genes. This procedure of carrying out mutagenesis at a targeted site in the genome is called site-directed mutagenesis. It involves the isolation of the DNA of the specific gene and the determination of the DNA sequence. It is then possible to construct a modified version of this gene in which specific bases or a series of bases are changed. The modified DNA can now be reinserted into the recipient cells and the mutants selected. Site-directed mutagenesis has found valuable application in improving strains, by enhancing the catalytic activity and stability of commercial enzymes, for example, penicillin G amidase.

Since the mid-1970s the synergistic use of classic techniques along with rational selection and recombinant DNA has made a significant impact in developing improved strains. Fermentation processes for products as diverse as human proteins and antibiotics and other therapeutic agents (chymosin, lactoferrin) have benefited from these combinatorial approaches. Transcription, translation, and protein secretion, activation, and folding are one or more of the rate-limiting steps critical for the overproduction of such therapeutic proteins. Achieving overproduction of active therapeutic proteins in bacterial or fungal heterologous gene expression systems has been made amenable due to the mix of classic and rational selection procedures. Genetic engineering along with classic methods has been used on numerous occasions to improve the performance of yeast and bacteria in alcohol fermentation, expand the substrate range, enhance the efficiency of the fermentation process, lower by-product formation, design yeast immune to contamination, and develop novel microbes that detoxify industrial effluents. Cost-effective production by fermentation of alcohols (ethanol, butanol) that can be used as substitutes for fossil fuels has been aided by this technology. Bacterial manufacturing of large quantities of hormones, antibodies, interferons, antigens, amino acids, enzymes, and other therapeutic agents to combat diseases has also become possible by recombinant DNA technology and strain improvement programs. Through increased gene dosage, improved efficiencies of antibiotic production have been achieved to relieve one or more rate-limiting steps. Novel and hybrid antibiotics and bioactive compounds have also been produced by combining different biosynthetic pathways in one organism that would have been difficult or impossible to manufacture through synthetic chemistry (e.g., *Cephalosporium acremonium* and *Claviceps purpurea*). Moreover, using recombinant DNA techniques, entire sets of genes for antibiotic biosynthesis have been cloned into a heterologous host in a single step. By cloning portions of the biosynthetic genes from one producer to another strain, hybrid compounds have also been synthesized, with novel spectra of activities and pharmacological applications. An example of this is the production by *Streptomyces peucetius* subsp. *caesius* of adriamycin (14-hydroxydaunomycin), an antitumor antibiotic.

Occasionally, it has been found that certain improved mutants produce extremely high levels of a specific enzyme. When analyzed, these mutants had multiple copies of a structural gene coding for the specific enzyme of interest. Increasing the number of gene copies in the cell (through gene cloning) has therefore been employed to overproduce enzyme precursors and their end product. In addition, mutations at the promoter or regulatory site have been demonstrated to alter secondary metabolite productivity. For example, in *Saccharopolyspora erythraea*, specific mutations at a ribosomal RNA operon terminator site altered the transcription and expression of the erythromycin gene cluster, and strains harboring these mutations overproduced enzymes involved in the later steps of erythromycin biosynthesis.

Once an improved strain is confirmed through bench-work studies, additional efforts are necessary to validate its performance. It is normally purified by reisolation, and the reisolates are verified for strain variability, homogeneity, and performance. They are preserved in large lots for examination under pilot plant conditions before being introduced for large-scale production.

Improved Strain Performance Through Engineering Optimization

Major improvements in fermentation are no doubt attributed to superior strains created through mutation or genetic alterations. Further improvement in culture performance can also be achieved by giving a strain the optimum environmental and physical conditions. During the strain improvement process, it is important to keep in mind that the ultimate success also depends on optimization of fermentation design factors. The use of batch or fed batch, continuous or draw-and-fill operation, the extent of shear, broth rheological properties, and oxygen and heat transfer characteristics all contribute to improvement in strain performance. The application of biochemical engineering principles can be used to design environmental parameters that shift kinetics of metabolic routes toward the desired product.

Improving Strain Performance Through Optimizing Nutritional Needs

The environment in which the altered strain is grown is known to influence higher product yield and get the best performance out of the culture. Since the media commonly used for production are different from the ones in which mutants are screened, media optimization is requisite to achieving the best response from the improved strains when scaled up to production. The media for production are reformulated so that they meet all growth requirements and supply the required energy for growth and product synthesis. Early bench work is typically performed with biochemically defined media to elucidate metabolite flux and regulation (inhibition or repression) by specific nutrients and physical variables. Later research is done to develop complex media that are more cost-effective to support cultural conditions of improved strains and maximize product synthesis without producing additional impurities that may impact isolation of the product. Additional issues, such as inoculum media and transfer criteria, media sterilization, pH, cultivation variables, and the sensitivity of the culture to different batches of raw material, are addressed during media optimization.

Statistical computer-based methods and response surface modeling are available for the study of many variables at the same time. A full search is normally made of every possible combination of independent variables to determine appropriate levels that give the optimum response in strain performance. Success in this area can be enhanced if additional physiological data are available, such as the role of precursors, the steps in the biosynthetic pathway, carbon flux through the pathway, and the regulation of primary and secondary metabolism by carbon and nitrogen. Controlling the levels of metabolites and precursors during fermentation aids in controlling lag and repression or toxicity effects. The removal of inhibiting products has been practiced where increasing the concentration up to economical levels demonstrates poor process kinetics. Adding chelating agents has been beneficial if the fermentation is found to be sensitive to substrate-specific repression. Further improvement in strain performance and productivity gain has been observed when the key enzymes participating in product formation are stablilized. For example, biosynthesis of the antibiotic gramicidin has been improved greatly by adding precursor amino acids that are substrates for the key enzymes.

Influence of Bioengineering in Improving Strain Performance

The ultimate destination of an improved strain is a large fermenter in which the desired product is made for commercial use. Conversion of laboratory processes to an industrial operation is called scale-up. It is not a straightforward process, requiring the use of methods of chemical engineering, physiology, and microbiology for success. The goal of the scale-up team is to cultivate improved strains under optimum production conditions. Open communication, data feedback, and synergy between engineers and scientists are vital to facilitate successful launch and scaling up of new and improved strains. Factors such as media sterilization for culture seed and production, methods of aeration and agitation, power input, control of viscosity, and evaporation rates are considered when moving new strains into production. In addition, sterility factors, heat transfer, impeller types, baffle types and positioning, the geometry and symmetry of the fermenter, mixing times, oxygen transfer rates, respiratory quotient and metabolic flux, disengagement of gases, and culture stability are all important in bringing the improved mutants from the laboratory- to industrial-scale production. Furthermore, metabolic feeds and the impact of the addition of feeds subsurface or surface, as well as timing of additions, are also optimized for directing ways toward the desired product. In some cases, process control and parameter optimization are facilitated using near-infrared spectroscopy and Fourier transform infrared spectroscopy when integrated in the fermentation process. This allows fermentation broth analysis and the ability to assay *in situ*, avoiding sample preparation and permitting timely adjustment of environmental parameters. However, online mass spectroscopy analysis is helpful mostly in a fermentation that is less sensitive to specific variables, as it cuts down routine assay work, sample preparation time, and the need for expensive equipment.

After the successful introduction of the improved strain in fermenters, production processes are validated and designed to run automatically for comparative and consistent operations. In some cases mathematical modeling of the physiological state and microbiological process are elucidated for maximizing strain performance. Typically, this is done in three stages: (1) qualitatively analyzing relationships among growth, substrate consumption, and production (usually based on the assumption of metabolic pathway and biogenesis of product); (2) establishing mathematical formulations and kinetic equations of the model, emphasizing the role of operator functions and technical operation associated with overproduction; and (3) estimation of parameters and simulation of the model on the basis of experimental data. During these scale-up and modeling studies, emphasis is also placed on the capital and operating costs as well as on the reliability of the process.

Among the various strains of microbes that have been scaled up, a few problems have invariably been noticed when scaling up filamentous organisms. The viscous nature of the culture creates heterogeneity, uneven mixing and distribution of bubbles, and failure to disperse micelle and floc formation. Several of these factors can be addressed up front during strain selection. The development of morphological mutants with short mycelia (higher surface area per unit volume) has been beneficial. This change can also influence the release of heat and spent gas without causing gradients in the fermenters. In addition, methods of mixing, nutrient feeding, and pH control also play critical roles in successful scale-up of improved mycelial strains. Finally, data on performance of the broth in pilot and large-scale purifications are also crucial for approval of improved strains for market production.

Metabolic Engineering for Strain Development

In the broadest sense, metabolic engineering is a new technology in strain improvement that optimizes, in a coordinated fashion, the biochemical network and metabolic flux within the fermenters, with inputs from chemical engineering, cell physiology, biochemistry, and genetics. By systematically analyzing individual enzymatic reactions and pathways (their kinetics and regulation), methods are designed to eliminate bottlenecks in the flow of precursors and to balance stoichiometrically the distribution of metabolites for optimum product formation. Nuclear magnetic resonance studies of metabolic flux analysis and kinetic measurements are further combined with thermodynamic analysis of the biological process to predict better strain performance. The principles governing a biosynthetic pathway, including genetic controls, interaction with complex raw material sources, and bioreactor operations and mathematical modeling strategy, are exploited to exceed the microbe's capability and improve its productivity. Metabolic engineering applications in strain improvement have found a special niche as a result of their previously observed successes in the production of amino acids and biopolymers from strains of *Brevibacterium*, *Corynebacterium*, and *Xanthomonas*.

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Streptococcus Pneumoniae

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Abbreviations

CbpA	choline-binding protein A
CibABC	competence induced bacteriocin
CSP	competent stimulating peptide
DCC	day care center
EUROPNEUMO	European Meeting on the Molecular Biology of the Pneumococcus
IPD	invasive pneumococcal disease
ISPPD	International Symposium on Pneumococci and Pneumococcal Disease
LytA	autolysin A
MLST	multilocus sequence typing
NanA	neuraminidase
NET	neutrophil extracellular trap
Pal	pneumococcal bacteriophage lytic enzyme
PBP	penicillin-binding protein
PFGE	pulsed-field gel electrophoresis
Ply	pneumolysin
PMN	polymorphonuclear leucocytes
PspA	pneumococcal surface protein A
SrtA	sortase A

Defining Statement

The primary (if not only) natural habitat of *Streptococcus pneumoniae* on this planet is the nasopharynx of preschool-age children, and antibiotics and vaccines not only combat pneumococcal disease but also drive the evolution of drug-resistant and novel capsular types of this species. In this sense, humans are not only targets but also evolutionary partners of *S. pneumoniae* as well.

Guides to the *S. pneumoniae* Literature

Useful guides to the rapidly expanding literature on various aspects of the microbiology and infectious diseases of *Streptococcus pneumoniae* may be found in books listed at the end of this article which cover contributions to the field in the early and the more recent era.

Periodic updates on progress are also available through informal meetings of two groups of scientists: those interested primarily in pneumococcal molecular biology (EUROPNEUMO: European Meeting on the Molecular Biology of the Pneumococcus) and those interested in pneumococcal disease (ISPPD: International Symposium on Pneumococci and Pneumococcal Diseases). The two groups meet in alternate years at various worldwide locations. The last meeting of EUROPNEUMO was in April 2007 in Lisbon, Portugal – organized by Hermínia de Lencastre and Alexander Tomasz. The latest meeting of ISPPD was in June 2008 in Reykjavik, Iceland, organized by Ingileif Jonsdottir.

Pneumococcus as a Pathogen and as a Model Microbe for Molecular Biology

> *Pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death*
>
> *(Benjamin White, 1938 in: The Biology of Pneumococcus)*

S. pneumoniae was first described in 1881 by Pasteur and Sternberg in independent observations. In the same decade, this Gram-positive pathogen with lancet-shaped cells that grow in most media in pairs or short chains of 'diplococci' was recognized as a major cause of infections that included pneumonia, meningitis, otitis media, and endocarditis. *S. pneumoniae* routine identification is done through the alpha hemolysis surrounding colonies obtained on blood agar, negative reaction with catalase, susceptibility to optochin, and solubility of the bacteria in bile salts. Most *S. pneumoniae* isolates are shielded by a polysaccharide capsule that hinders phagocytosis. At least 91 different capsules have been described, and serologic typing (serotyping) remains one of the most frequently used methods for the characterization of pneumococcal isolates.

Since its discovery, this bacterium has been the subject of intensive studies as a cause of major and often life-threatening human infections. While the primary aim of these studies was the control of pneumococcal disease, the same efforts have also lead to seminal scientific discoveries in the laboratory which included the identification of the pneumococcal polysaccharide antigens as vaccines, the ability of capsular polysaccharides to induce antibodies, the discovery of bacterial gene transfer which led to the identification of the 'transforming principle' (later named DNA) as the genetic material. Efforts to degrade the capsular polysaccharide surrounding the pneumococcus have led to the first use of an 'enrichment culture'. The remarkable and rapid dissolution of pneumococci by bile led to the identification of the first bacterial autolytic enzyme. Pneumococcus was among the first pathogens in which the therapeutic efficacy of the newly discovered penicillin was tested. The role of the polysaccharide capsule providing resistance against phagocytosis was identified, and studies on the rapid fluctuations in the pneumococcal capacity to take up DNA from the medium and undergo genetic change has led to the identification of the first bacterial quorum sensing factor.

Thus, efforts to understand and control pneumococcal disease went hand in hand with some of the fundamental discoveries of molecular biology. Interestingly, many of these phenomena first discovered in the *in vitro* world of the microbiology laboratory were subsequently identified as major factors driving the evolution of new pneumococcal lineages in the real life world of pneumococcal colonization, infection, and disease.

The most promising – and ambitious – current efforts to understand the impact of antibiotics or vaccines on the pneumococcal colonization, infection, and disease in humans are directed toward combining carefully designed epidemiological studies with the characterization of pneumococcal isolates by high resolution molecular techniques developed in the molecular biology laboratory.

Burden of Pneumococcal Disease

In the preantibiotic era, pneumococcal pneumonia was so common and fatal that it was termed as the "old man's friend" and the "captain of the men of death" by William Osler. In the late 1990s, before the introduction of the first pneumococcal seven-valent conjugate vaccine in the United States, data from the Centers for Disease Control and Prevention estimated the annual frequency of pneumococcal infections as 3000 cases of meningitis, 50 000 cases of bacteremia, 500 000 cases of pneumonia, and 7 million cases of otitis media and an estimated mortality of about 40 000 deaths per year.

While no similar dependable estimates are available from the less-developed countries of the world, evaluation of the impact of a nine-valent conjugate vaccine in one randomized control trial conducted in Africa clearly indicated that pneumococcal disease is a major contributor to the mortality of children in African countries.

In Gambia, 77% (95% CI, 51–90%) efficacy against vaccine-type invasive pneumococcal disease (IPD) was found with a 50% (95% CI, 21–69%) efficacy against all types of IPD. Furthermore, a 16% (95% CI, 3–28%) decrease in all-cause mortality was found among vaccinated children. In South Africa, the efficacy of the nine-valent pneumococcal conjugate vaccine against IPD among HIV-negative children was 83% (95% CI, 39–97%) and corresponding figures were 65% (95%CI, 24–86%) among HIV-positive children.

A report in 2007 from the WHO estimated that 1.6 million people continue to die every year due to pneumococcal disease including 0.7–1 million children aged less than 5 years, the majority living in developing countries, where the pneumococcal conjugate vaccine is not available. The burden of disease associated with the elderly in these countries remains to be defined.

In addition to young children and the elderly, individuals of all ages infected with HIV are at a substantial higher risk of serious pneumococcal infection. For example, the risk of pneumococcal pneumonia is 25-fold higher among HIV-infected people compared to HIV-uninfected people. The incidence of pneumococcal invasive disease among HIV-positive children is 9–43 times higher than among HIV-negative children and the rates of IPD among HIV-positive adults are 6–343 times higher than among HIV-negative adults.

Pneumococcal Infection and Viral Disease

In the era of preparedness for an anticipated new influenza or bird flu pandemic, the well-documented contribution of pneumococci in the mortality associated with flu becomes increasingly important.

Several lines of evidence have highlighted that secondary infections by pneumococci in patients with viral respiratory disease can have devastating consequences. Studies conducted during the 1918 influenza epidemic, which is estimated to have led to at least 40–50 million deaths, demonstrated that an important fraction of the deaths took place 2 weeks after the onset of influenza symptoms, suggesting that superinfection by a common bacterial respiratory pathogen had occurred. Direct evidence supporting the role of pneumococcal secondary infection leading to fatal pneumonia has been described.

In a double-blind, randomized, placebo-controlled trial of a nine-valent pneumococcal conjugate vaccine in South Africa, it was found that the vaccine prevented 31% of virus-associated pneumonia in hospitalized children, suggesting that an important fraction of virus-associated pneumonia among hospitalized children was attributable to bacterial coinfection that could be prevented by bacterial vaccines.

Experiments on modeling viral–bacterial infection in animals showed that if a mouse model was challenged with a nonlethal dose of influenza virus and approximately 7 days later was challenged with pneumococcus, 100% mortality occurred. This effect was specific to viral infection preceding bacterial infection. Together these data strongly suggest that pneumococcal vaccination could have a beneficial role in preventing influenza-associated mortality in the advent of a new influenza pandemic.

The Natural Reservoir of *S. pneumoniae*

Humans are not only the target of diseases caused by *S. pneumoniae* but are also the primary ecological reservoir of this bacterial pathogen – although two anecdotic studies have found carriage of pneumococci by horses and isolation from wild chimpanzees. Thus, interventions to combat pneumococcal disease such as the introduction of antibiotics or vaccines also impact on the human nasopharyngeal flora of pneumococci selecting for drug-resistant lineages and strains with less common capsular types. In this sense, humans are also evolutionary partners of this microbe and many – if not all – of the genetic events that allow these bacteria to borrow pieces of foreign DNA to remodel their penicillin-sensitive enzymes, acquire mobile elements that confer antimicrobial resistance to different classes of antibiotics, or undergo capsular switches to evade the action of vaccines targeting the capsule most likely occur in pneumococcal populations that inhabit the human nasopharynx, more specifically, the nasopharynx of preschool-age children. The latter, for reasons not fully understood, are the primary carriers of this bacterial species. For these reasons, the day care centers (DCCs) in which preschool-age children are now recruited in many of the countries of the developed world have become major foci of epidemiological studies of pneumococci.

Indeed, several studies have shown that children of preschool age are the major reservoir of pneumococci and by school time a spontaneous decrease in carriage occurs. The mechanism of extensive colonization in infancy and the loss of carriage with age are not well understood. It is also well established that in infancy the most dominant capsular types colonizing the nasopharynx are typically of the serogroup 6, 9, 14, 19, and 23 in countries across the world. This commonality of serotypes colonizing the young host contrasts with the well-documented and sometimes extensive differences in the serotypes of pneumococci that most frequently cause invasive disease in various parts of the world. The mechanism of geographic variation in serotype abundance and their change in time is not known.

An important source of problems contributing to the difficulties of interpretation of many aspects of pneumococcal epidemiology is the way pneumococcal colonization has been routinely assayed in the overwhelming majority of the studies conducted so far. In most studies, a single colony recovered on blood agar plates from the nasopharyngeal swabs is assumed to represent the entire colonizing flora. However, simultaneous carriage of multiple strains of pneumococci in the nasopharynx has been known for several decades and was documented in early studies conducted in the 1930s and 1940s, which used mouse inoculation assays to detect the strains. As these methods were very labor-intensive and expensive they have been abandoned. More recent studies in which a number of colonies were picked from the primary blood agar plates and were characterized clearly showed that the nasopharyngeal flora is heterogeneous: it may consist of more than one strain of pneumococci; some representing the majority, others – often present with lower frequencies – may be of completely different serotypes and molecular type (**Table 1**). Certain pneumococcal serotypes such as serotypes 1 and 5 that can be recovered from disease sites but have seldom been seen in the nasopharynx may represent such minority residents in the nasopharyngeal flora. The same minority clones may be the source of the novel pneumococci emerging after the introduction of the conjugate pneumococcal vaccine.

Multiple pneumococcal carriage is apparently more abundant among populations with high pneumococcal carriage rates such as children from Papua New Guinea, Gambia, or Australian Aborigines. Multiple carriage rates in the range of 20–30% have been reported among these populations. Among other children, typical rates of multiple carriage have been in the range of 5–10%.

Table 1 Properties of multiple isolates obtained from nasopharyngeal samples containing two strains of *S. pneumoniae*

Sample	*Isolate code*	*Serotype*	*MIC* (μg *ml*$^{-1}$) to penicillin	*Antibiotype (resistant to)*	*PFGE type*	*PFGE-lytA (kb)*	*Addition mitomycin C*	*Phage DNA*	*comC allele*
A	106	11	0.016	E, Cc	SSS	85	No lysis		*comC*1
A	106-1	6B	0.023	E, Cc, Te	M	280, 110, 90	Lysis	Yes	*comC*1
A	106-2	11	0.006	E, Cc	SSS	85			
A	106-3	11	0.008	E, Cc	SSS	85			
A	106-4	11	0.008	E, Cc	SSS	85			
A	106-5	11	0.008	E, Cc	SSS	85			
A	106-6	6B	0.016	E, Cc, Te	M	280, 110, 90			
B	325	19F	0.047	–	H	90, 35	No lysis		*comC*2.1
B	325-1	NT	0.5	–	TTT	170, 90, 40, 30	ND		*comC*2.1
B	325-2	NT	0.75	–	TTT	170, 90, 40, 30			
B	325-3	NT	1	–	TTT	170, 90, 40, 30			
C	448	19A	0.094	SXT	D	235, 90, 80	Lysis	Yes	*comC*1
C	448-1	19A	0.064	SXT	D	235, 90, 80			
C	448-2	23F	2	C, Te, SXT	A	230, 100, 40	Lysis	Yes	*comC*2.2
C	448-3	19A	0.094	SXT	D	235, 90, 80			
C	448-4	19A	0.094	SXT	D	235, 90, 80			
C	448-5	19A	0.094	SXT	D	235, 90, 80			
C	448-6	19A	0.064	SXT	D	235, 90, 80			
D	541	6B	0.023	Te, SXT	M	280, 90, 60	Lysis	Yes	*comC*1
D	541-1	19A	0.023	SXT	UUU	90	No lysis		*comC*1
D	541-2	19A	0.023	SXT	UUU	90			
D	541-3	6B	0.016	Te, SXT	M	280, 90, 60			
D	541-4	6B	0.012	Te, SXT	M	280, 90, 60			
D	541-5	6B	0.008	Te, SXT	M	280, 90, 60			

C, chloramphenicol; E, erythromycin; Cc, clindamycin; Te, tetracycline; SXT, sulfamethoxazole-trimethoprim.

© American Society for Microbiology. Reproduced from Sá-Leão R, Tomasz A, Santos Sanches I, and de Lencastre H (2002) Pilot study of the genetic diversity of the pneumococcal nasopharyngeal flora among children attending day care centers. *Journal of Clinical Microbiology* 40: 3577–3585.

Stages in Pneumococcal Pathogenesis: Virulence Factors and Host Defense

Virulence in pneumococci is multifactorial and involves complex and multiple interactions with the host. Well over 300 genes have been implicated in virulence in at least one animal model of pneumococcal infection. A genome-wide screen – by signature-tagged mutagenesis – of the reference strain TIGR4 for genes essential for lung infection in an animal model identified close to 400 genes. Individual putative virulence factors appear to play a variety of roles – both 'aggressive' as well as 'defensive' at various stages of pneumococcal infections. Some of the pneumococcal virulence factors, such as the capsular polysaccharide, the major autolysin LytA, the intracellular toxin pneumolysin (Ply), and the sortase A (SrtA), are present in virtually all pneumococcal isolates. Other important virulence determinants, such as the recently described pilus operon, also play important roles in virulence but these determinants seem to be associated with specific clones of pneumococci. Secreted DNAase appears to have a role in escaping the neutrophil extracellular traps (NETs) and pneumococcal resistance to the widely spread cationic antimicrobial peptides appears to involve incorporation of D-alanine esters into the pneumococcal cell wall teichoic acids.

Host factors 'matching' in numbers the virulence genes of the pneumococcus have also been identified using a variety of models. These factors cover a wide range of functions: pattern recognition elements that are components of the innate immune system as well as host defense factors more specific for the invading pneumococcus such as the scavenger receptor involved with lung defense or the generation of adaptive immunity directed primarily against the capsular polysaccharide.

Capsular Polysaccharides

While rare *S. pneumoniae* isolates free of the capsule do exist the overwhelming majority of clinical strains express one capsular polysaccharide attached to the pneumococcal cell wall. Nonencapsulated strains, for instance, strains R6 or R36A, which are most frequently used in laboratory studies on *S. pneumoniae*, are completely avirulent in animal models of disease. The role of the capsular polysaccharide in pneumococcal virulence appears to be the inhibition of complement-mediated opsonophagocytosis by macrophages or polymorphonuclear leucocytes (PMN). Except for the capsular polysaccharide types 3 and 37, all the other polysaccharide capsules appear to be covalently linked to the pneumococcal cell wall. Pneumococci have an enormous genetic repertoire to produce – potentially – as many as 91 chemically different polysaccharide chains. A unique feature of the structure of capsular loci is that type-specific genes are flanked by common determinants, thus allowing for a relatively easy exchange of genetic determinants within this region of the pneumococcal chromosome. The frequent 'capsular switch' that can appear spontaneously or is driven by vaccine pressure among clinical isolates appears to be the consequence of a relatively easy genetic change at the capsular loci. In such cases, isolates that share common genetic backgrounds (defined by multilocus sequence typing (MLST) or pulsed-field gel electrophoresis (PFGE) profile) appear to differ only in the type of capsule at their surface.

Cell Walls in Virulence

The unique choline component of the wall and membrane teichoic acid appears to perform multiple roles both in the physiology of the pneumococcus and also as an interactive component with the host. The species of *S. pneumoniae* is unique in that it requires choline as an essential nutrient for growth. Recently, pneumococcal strains in which this auxotrophic requirement for choline is lost have been isolated. In one of these isolates point mutations in one of the choline utilization genes – *tacF* – have been identified as the mechanism responsible for the choline-independent growth. TacF, a teichoic acid flippase, was proposed to catalyze the transfer of teichoic acid chains across the pneumococcal plasma membrane. A second choline-independent strain was recovered from a heterologous genetic cross in which *Streptococcus oralis*, a bacterial species that contains choline in its teichoic acid but does not require choline for growth, served as the DNA donor and the recipient was the R6 strain of *S. pneumoniae*. The mechanism of choline independence in this strain called R6Cho$^-$ appears to be different from the mechanism identified in the other choline-independent mutants.

Recent studies have shown that pneumococcal constructs capable of growing without choline and expressing the capsular polysaccharide type 2 on their surface have severely reduced virulence potential in several animal models of pneumococcal disease and are also inhibited from colonizing the nasal epithelium of mice. The mechanism of this striking impact of the teichoic acid choline units on pneumococcal virulence is not understood to date.

Pneumococcal cell wall components were shown to induce the production of preinflammatory cytokines both in the murine intraperitoneal models and in the rat and rabbit models of meningeal disease. Structural features of the peptidoglycan involved with the recognition by the innate immunity system have been identified in the Drosophila model. At least two pneumococcal cell wall-modifying enzymes: PGDA, a peptidoglycan glucosamine deacetylase and PCE, a phosphoryl choline esterase, were shown to play roles in virulence as indicated by the impact of inactivation of the corresponding genetic determinant on virulence.

Regulation of Virulence

The expression of capsular polysaccharides appears to be regulated: contact with epithelial cells was shown to cause suppression in the amounts of capsule, which can subsequently increase once the bacteria have reached the blood stream.

Different modalities of growth. Recent studies have shown that pneumococci can grow in two different modalities: as planktonic cells typical of bacteremic infections and as biofilms typical of meningitis. Different sets of genetic determinants matching these two different growth styles of pneumococci are expressed.

Opaque versus transparent phenotype. In 1994, Weiser and colleagues described that pneumococci could undergo phase variation, that is, changes in properties of the cell surface that could lead to two different colony phenotypes: opaque and transparent. Spontaneous, reversible variation between the two colony phenotypes with a frequency ranging from 10^{-3} to 10^{-6} per generation has been described. This frequency appears to be independent of *in vitro* growth conditions.

The same authors showed that the opaque phenotype was associated with increased amounts of capsular polysaccharide and pneumococcal surface protein A (PspA), whereas transparent variants were associated with increased amounts of choline-binding protein A (CbpA) and autolysin A (LytA). Opaque colonies were described as dome shaped and large contrasting with transparent colonies, which were umbilicated, suffered quicker autolysis, and had a higher efficiency of natural transformation. Cultures obtained from opaque colonies had decreased binding to serum C-reactive protein, decreased opsonophagocytosis, and increased virulence in systemic infections. By contrast, transparent colonies had increased adherence, and colonized more efficiently in an animal model of carriage.

More recently, DNA microarray strategies have identified an additional number of genes that appear to have differential regulation between isogenic pairs of strains displaying opaque or transparent phenotypes. In particular, the transparent phenotype was associated with increased production of neuraminidase (NanA). Studies on the fatty acid composition of pairs of opaque and transparent colonies found a lower degree of unsaturated fatty acids in the opaque variants.

A single large study has attempted to examine the prediction that opaque variants are associated with increased pathogenesis by looking at the colony phenotypes displayed by a large collection of invasive disease isolates with a low number of *in vitro* passages. This collection of 304 isolates included representatives of ten serotypes displaying genetically diverse backgrounds. The authors confirmed that the opaque phenotype dominated among invasive disease isolates but also noted a previously unreported association between serotype and colony phenotype. This observation led them to suggest that the association between the opaque phenotype with particular serotypes might contribute in part to explain the observed differences in the invasive disease potential of certain serotypes.

Contribution of Genotype and Serotype to the 'Invasive Disease Potential'

The introduction of molecular typing techniques for the characterization of *S. pneumoniae* recovered from disease and from colonization sites combined with extensive epidemiological studies has initiated efforts to better define the contribution of serotype versus molecular type to the 'invasive' potential of a pneumococcal strain. The frequent representation of the so-called pediatric serotypes (primarily 6B, 14, 19F, and 23F) in the nasopharyngeal flora of children clearly provides an increased opportunity for strains expressing these serotypes to invade during periods of decreased host defense. Thus, while assigning a true 'invasive disease potential' to a strain, the odds ratios expressing the frequency of recovering the particular strain from colonization versus infection sites must be taken into account.

The relative contribution of serotype versus genotype to the invasive disease potential is a currently unsettled issue. Although it is relatively consensual that the capsular type expressed is extremely important for the disease potential of a particular strain, there are studies suggesting that the genetic background of that strain is also important, which, after all, is in agreement with the finding of several genetic determinants essential for full virulence of pneumococci.

A further potential problem contributing to the difficulties of interpretation of these empirical estimates of invasive disease potential of serotypes and clones of pneumocooci is the way pneumococcal colonization has been traditionally studied ignoring multiple colonization (discussed above).

The relative contribution of the host versus the pathogen to the occurrence of pneumoccal infection is debatable but clearly the host plays a very important role. Recent studies have started to identify genetic polymorphisms in the determinants implicated in the host immune response including some that are associated with susceptibility to IPD and others that confer a protective effect to it.

Human Intervention

There have been at least three important interventions by humans that radically altered the *in vivo* landscape and epidemiology of *S. pneumoniae*: (1) the introduction of antibiotics into therapeutic practice, (2) the introduction of the conjugate antipneumococcal vaccine, and (3) the proliferation of DCCs in the developed part of the world.

The introduction of antibiotics did not change the incidence of pneumococcal infections; it contributed significantly to the control and outcome of pneumococcal disease but has also led to the emergence of antibiotic-resistant strains.

The introduction of a conjugate antipneumococcal vaccine in the United States had major impact on the incidence of IPD. However, the rate of pneumococcal carriage did not decrease and the frequency of antibiotic-resistant strains among colonizing pneumococci has initially decreased but is now increasing due to the expansion of pneumococcal nonvaccine capsular serotypes that are also resistant to antibiotics.

The institution of day care has emerged as a unique epidemiological entity in which many children of preschool age are cohorted. The high carriage rate of respiratory pathogens, immunological status, and typical child behavior, together with the frequent occurrence of viral respiratory diseases and the extensive and often imprudent use of antimicrobial agents among this age group, are the most likely reasons why attendance at DCCs has become a risk factor for both carriage and infection by antibiotic-resistant strains of pneumococci.

Antibiotic Resistance and Insights Provided by Molecular Typing

The first penicillin-resistant strain of *S. pneumoniae* that appeared in the clinical environment and invoked comments in the infectious diseases literature was the strain isolated from the throat of a healthy child in the mid 1960s in a remote village, Agunganak in Papua New Guinea. Although initially the possibility of geographic spread of the penicillin-resistant pneumococcus was judged remote, this prediction was soon contradicted by the massive outbreak of pneumococcal disease in South African hospitals in 1977, which was caused by multidrug-resistant strains of this bacterium. Between the early 1980s and the late 1990s reports on the detection and increase both in frequency and in antibiotic resistance level of drug-resistant strains of *S. pneumoniae* have appeared in increasing numbers and the antibiotic-resistant pneumococcus has become a global phenomenon that began to make effective chemotherapy sometimes problematic.

Global Spread of a Few Pandemic Clones of Penicillin-Resistant Pneumococci

Introduction of pneumococcal typing techniques such as PFGE or MLST has clearly shown that among the very large number of lineages of resistant pneumococci a handful of highly epidemic clones emerged and achieved massive and often pandemic spread. The most outstanding of these is Spain^{23F} ST81 originally called the 'Spanish/USA clone' usually expressing serotype 23F and carrying resistance to penicillin, tetracycline, and chloramphenicol and often to erythromycin and sulfamethoxazole–trimethoprim.

An apparent intercontinental transfer of this clone from Southern Europe to the United States was demonstrated. This clone was subsequently identified in numerous national and international surveillance studies, both as a powerful colonizer and also as a strain capable of causing the entire spectrum of pneumococcal diseases among both adults and children.

Similar importation of a multidrug-resistant pneumococcal clone, the penicillin-resistant clone Spain^{6B} ST90 presumably from Southern Europe to Iceland was demonstrated in the early 1990s. This so-called 'Icelandic' clone expressing serotype 6B and resistance to penicillin, tetracycline, chloramphenicol, erythromycin, sulfamethoxazole–trimethoprim, and occasionally to third-generation cephalosporins as well, quickly spread in Iceland and within 3 years of its detection was shown to be responsible for close to 20% of all pneumococcal disease in that country.

A third genetic lineage France^{9V} ST156 originally referred to as the 'French/Spanish' clone carries resistance to penicillin and tetracycline and occasionally to sulfamethoxazole–trimethoprim and typically exists in two capsular serotypes: 9V or 14. This clone was shown to have spread in Europe, Latin America, USA, Canada, and Asian countries (reviewed at the Pneumococcal Molecular Epidemiology Network (PMEN), website available at www.sph.emory.edu/PMEN/).

The introduction and widespread use of molecular typing techniques has led to the establishment of an international depository and 'clearing' house for characterized *S. pneumoniae* clones, the so-called PMEN. This platform has also become useful in providing a uniform set of rules to name new clonal lineages, register both their molecular and epidemiological characteristics, serotypes, isolation dates and sites and clinical sources as well (for more information visit www.sph.emory.edu/PMEN/).

Despite the fact that penicillin resistant strains have been isolated from countries all over the world, their incidence varies widely from country to country and from one geographic site to another.

The impact of antibiotic resistance on chemotherapy varies with the particular infection. Even low-level resistance to antibiotics requires change in chemotherapy in meningitis because of the low penetration of the cerebral spinal fluid by this class of antibiotics and because of the need for bactericidal concentrations. On the other hand, penicillin therapy was shown to remain effective in pneumococcal pneumonia caused by penicillin-resistant strains with MIC values as high as 1–2 $\mu g\,ml^{-1}$.

Penicillin Resistance: Genes and Phenotypes

The mechanism of penicillin resistance in pneumococcal clinical isolates is based on the remodeling of several of the genetic determinants – *pbp* genes that encode for proteins (penicillin-binding proteins, PBPs) that catalyze various stages in the pneumococcal cell wall synthesis. The process of remodeling involves recombinational events with fragments of *pbp* genes imported from heterologous species most often from *Streptococcus mitis*. These 'mosaic' *pbp* genes produce PBPs with decreased affinity for penicillin, which is the ultimate basis of the increased penicillin MIC values.

Examination of the cell wall chemical structure in penicillin-resistant clinical isolates showed additional profound abnormalities in these bacteria, specifically the increased representation of branched muropeptide components in their cell wall peptidoglycan. A genetic follow-up of these studies identified the new determinants, *murM* and *murN*, responsible for the biosynthesis and attachment of the two amino acid components that form the muropeptide branches. The *murM* and *murN* genes of resistant strains showed clear evidence of mosaicism, indicating the presence of DNA sequences of heterologous origin. Most interestingly, inactivation of *murM* caused a complete loss of penicillin resistance, which could be recovered in appropriate complementation experiments. A detailed biochemical mechanism of the synthesis of pneumococcal muropeptides and a mode of action for MurM and MurN proteins was recently described.

The critical nature of cell wall chemistry for the penicillin-resistant phenotype was further documented by the recent identification of yet another cell wall-modifying enzyme: a muramic acid *O*-acetylase. Inactivation of the structural gene named *adr* caused loss of penicillin MIC value, similar to the case of strains in which MurM was inactivated.

It seems that genetic determinants in addition to the mosaic *pbp* genes producing the low-affinity penicillin targets are also essential for optimizing the resistant phenotype. This scenario is reminiscent of the mechanism identified in methicillin-resistant *Staphylococcus aureus*, in which high-level resistance requires not only the central resistance determinant *mecA*, but a number of additional 'auxiliary determinants' as well in the genetic background of the bacteria.

Genetic Diversity among Penicillin-Resistant and Penicillin-Susceptible Pneumococci Causing Invasive Disease

Several studies have compared the genotypes of penicillin-resistant (MIC higher than $1\,\mu g\,ml^{-1}$) versus penicillin-susceptible isolates of pneumococci expressing the same serotypes and recovered from invasive disease. Studies with strains isolated from children in South American countries are illustrative of the common findings of such studies. The major and striking difference among the resistant and susceptible isolates was the relative genetic homogeneity of most resistant isolates, more than 80% of which were shown to belong to one of two pandemic penicillin-resistant clones: Spain^{23F} ST81 and/or France$^{9V/14}$ ST156 (**Figure 1**). In contrast, penicillin-susceptible isolates expressing the same serotypes and recovered from similar disease sites showed great genetic diversity (**Figure 2**). A similar – inverse – relationship between genetic diversity and penicillin MIC value has already been described in other epidemiological studies. These observations may reflect the expensive 'fitness' cost associated with the mechanism of pneumococcal penicillin resistance, which may be compatible only with a limited number of genetic backgrounds available in this bacterial species.

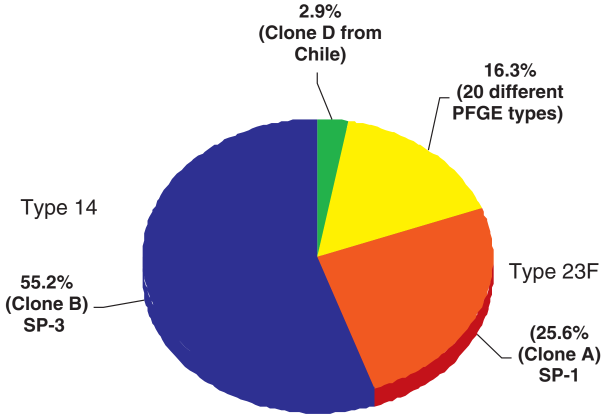


Figure 1 Pie chart showing the isolation percentages in Latin America of two penicillin-resistant pandemic clones versus all other clones from among 172 penicillin-resistant invasive isolates. Note that the two clone types A and B are responsible for ~81% of all pneumococcal infections. Clone A (serotype 23F) refers to the Spain^{23F} ST81 clone and clone B refers to the France^{9V} ST156 clone. Tomasz A, Corso A, Severina EP, *et al.* (1998) Molecular epidemiologic characterization of penicillin-resistant *Streptococcus pneumoniae* invasive pediatric isolates recovered in six Latin-American countries – an overview. *Microbial Drug Resistance* 4: 195–207.

Impact of the Seven-Valent Pneumococcal Conjugate Vaccine on Pneumococcal Disease and Carriage

In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7 targeting serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was licensed in the United States and soon after in many other countries worldwide. The vaccine was intended for children younger than 2 years of age. This vaccine was formulated to target the serotypes that cover over 80% of the cases of IPD among children younger than 5 years of age in the United States. Expected coverage rates in other countries, particularly in developing countries, are lower.

Following introduction of PCV7 in the United States, a reduction in the incidence of IPD occurred not only among young children but also in all other age groups due to a substantial indirect herd effect. In particular, a sharp decrease in IPD caused by vaccine types was observed, which was accompanied by a modest increase in IPD caused by nonvaccine types. By 2003 the total incidence of IPD among children <5 years of age had declined 75% from 96.7 cases per 100 000 population to 23.9, and among those aged 65 or older, a 29% decline was observed from 60.1 cases per 100 000 population to 41.7 cases.

The observed indirect effect of the vaccine is best explained by taking into account that the vaccine decreases

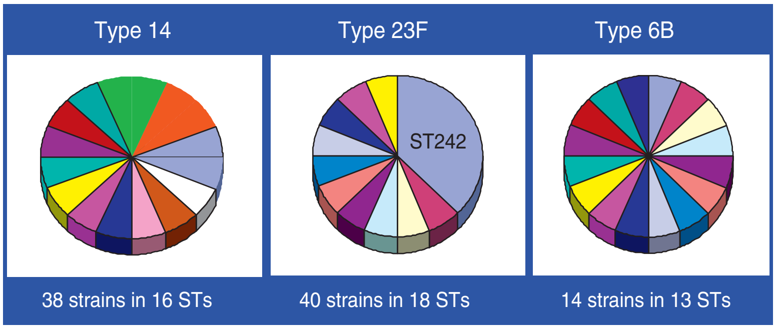
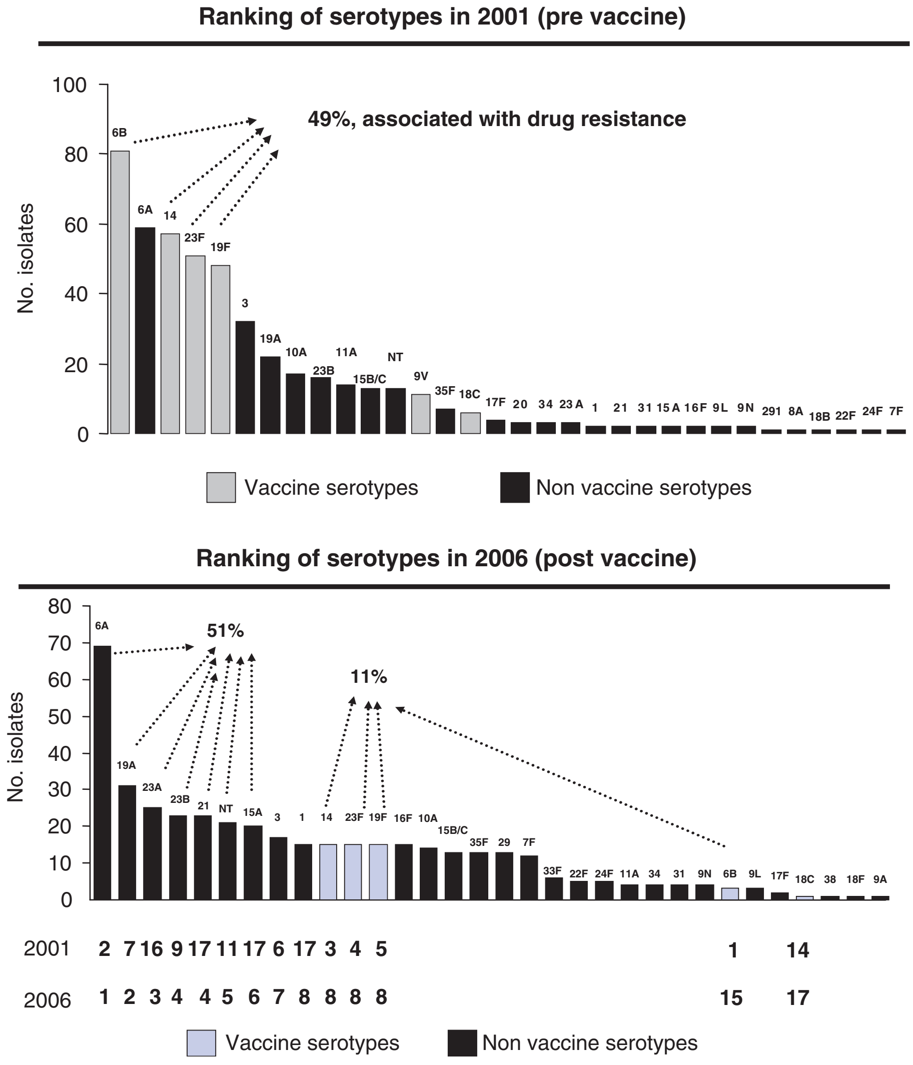


Figure 2 Pie charts (from left to right) showing the large number of different multilocus sequence typing (MLST) types (STs) for each of the three most prevalent capsular types (serotypes) 14, 23F, and 6B, which were associated with clinically invasive penicillin-sensitive pneumococcal disease in Latin America. Reproduced from Zemlickova H, Crisostomo MI, Brandileone MC, *et al.* (2005) Serotypes and clonal types of penicillin-susceptible *Streptococcus pneumoniae* causing invasive disease in children in five Latin American countries. *Microbial Drug Resistance* 11: 195–204.

carriage of vaccine types among vaccinees and these are the major reservoirs of pneumococci. By vaccinating young children, transmission of vaccine types has decreased substantially. An earlier study from Israel had demonstrated an indirect beneficial effect of PCV7 among children too young to be vaccinated whose siblings had received PCV7. The same study showed that pneumococcal colonization levels remain stable after vaccination due to a serotype replacement effect that results in the nasopharynx becoming mostly colonized by nonvaccine types.

An added bonus of the vaccine relies on the fact that most antimicrobial-resistant pneumococci isolated worldwide including those nonsusceptible to penicillin, and/or resistant to macrolides, or multidrug-resistant express mostly serotypes targeted by PCV7 (i.e., 6B, 9V, 14, 19F, and 23F). In agreement with these observations, a decline in antimicrobial-resistant pneumococci has been observed in the United States among isolates causing IPD. However, recent reports from the United States and elsewhere found that among colonizing isolates as well as those causing respiratory tract infections, levels of antimicrobial resistance among nonvaccine types are increasing, suggesting that the impact of PCV7 on antimicrobial resistance may be short-lived.

The phenomenon of serotype replacement and the short-lived effect of PCV7 on antimicrobial resistance are also beginning to be noticed in other countries that are using PCV7. For example, a recent study on the impact of PCV7 on pneumococci carried by Portuguese DCC attendees observed that serotype replacement has occurred among attendees regardless of their vaccination status (57% of the children had been vaccinated) (**Figure 3**). In other words, unvaccinated children were benefiting from being in contact with vaccinated children.



Ranking of serotypes in 2001 (pre vaccine)

Ranking of serotypes in 2006 (post vaccine)

Figure 3 Bar charts showing the number of clinical isolates recovered of each pneumococcal capsule type (serotype) before introduction (2001) of PCV7 (top) and after vaccine introduction (2006) in Portugal (bottom). All capsular types targeted by the vaccine decreased in recovery and most nonvaccine types increased in abundance. Reproduced from Sá-Leão R and de Lencastre H (2008) Personal communication.

Interestingly, rates of antibiotic resistance remained unchanged due to a balance between reduction of vaccine types and an increase in antimicrobial resistance among nonvaccine types.

Novel vaccines with greater valency – ten-valent (PCV7 plus 1, 5, 7F) and 13-valent (PCV10 plus 3, 6A, and 19A) are in development. These expanded valency vaccines will include the serotypes that are major causes of IPD in developing countries, such as the serotypes 1 and 5. A 13-valent conjugate vaccine will also target serotype 19A, which in the United States is now a major cause of IPD and is often multidrug-resistant. Universal protein vaccines, which should have broad coverage and low price,are currently in different stages of development.

Day Care Center Studies

DCCs have become increasingly frequent in the last decades in the developed world and their impact on infectious diseases has been discussed. In particular, several studies have focused on pneumococcal carriage among DCC attendees. Molecular epidemiological studies have shown that these 'novel' institutions play a crucial role in the transmission and amplification of pneumococcal lineages. Furthermore, marked differences in pneumococcal carriage rates across communities have been explained by differences in the proportion of children who attend DCCs in those communities.

Perhaps one of the largest surveillance initiatives of pneumococcal carriage among DCC attendees has been the one conducted in Lisbon and Oeiras, Portugal. The project began in 1996 and through the years, an ever-growing collection of isolates has been obtained. As of February 2008, more than 11 000 nasopharyngeal samples have been collected, resulting in the isolation of over 7600 pneumococci. Approximately 40% of the isolates were resistant to at least one commonly used antimicrobial agent. All drug-resistant isolates have been characterized by capsular type, antimicrobial susceptibility pattern, and DNA fingerprint generated after PFGE. In addition, a representative sample has been characterized by MLST. More than 40% of the fully susceptible pneumococci have also been characterized by the same combination of techniques. These and other studies have provided insights on the natural biology and the population structure of pneumococci, on the dynamics of the carrier state, and on the properties of DCCs as epidemiological entities. It was observed, for example, that DCCs are autonomous epidemiological units, which, at a given time, differ from each other in the pneumococcal population circulating among its attendees, even if the units are geographically close.

A longitudinal study showed that introduction of novel pneumococcal lineages occurred all year long in the DCCs and the fittest lineages were transmitted among attendees for several months, resulting in their amplification and providing ample opportunities for their spread in the open community as well.

Portuguese DCC attendees were found to be frequently colonized by pneumococci: in winter months an average of 65% of children were found to be carriers and virtually all children (98%) carried pneumococci at least once during a 1-year study. It was also shown that the nasopharyngeal flora of DCC attendees was a reservoir of internationally disseminated drug-resistant lineages capable of causing the entire spectrum of pneumococcal diseases, such as the successful clones Spain^{23F} ST81, Spain^{6B} ST9, and France^{9V} ST156.

Genetic Exchange *In Vivo*

Several of the intriguing phenomena first observed during studies of pneumococci in the microbiology laboratory also play important roles in the *in vivo* environment of this bacterium. DNA-mediated genetic transformation appears to be a major mechanism for acquiring antibiotic resistance genes and also for switching capsular type – as observed in epidemiological studies. The release of DNA through the activity of autolytic enzymes also appears to play an important role in the *in vivo* evolution of pneumococcal lineages.

Competence

Competence is a transitory state in which bacterial cells are able to take up exogenous DNA. This process may lead to genetic transformation if during the competent state there is exogenous DNA available in the environment – particularly if the DNA has high degree of homology to the DNA of the competent recipient cell. Nevertheless, heterologous DNA from other streptococcal species can also serve as 'donor' in recombinational events.

Pneumococcal competence has been studied for over four decades. However, it was during the past decade that major breakthroughs have been made in the elucidation of the molecular mechanisms leading to competence, the genes implicated in the competence regulon, and its role in pneumococci.

As of today most studies in this area are done based on a few well-characterized laboratory strains. Nonetheless, competence appears to be widely disseminated in pneumococci: a study on the distribution of two competence genes among a diverse collection of 214 clinical isolates found that both genes were ubiquitous in the collection.

The competent state is controlled by the early *com* genes *comABCDEWX*. Competence is triggered by accumulating exogenous levels of the 17 amino acid long pheromone CSP (competent stimulating peptide). The pre-CSP is 24 amino acid long encoded by *comC*, which is cleaved to CSP during export across the cytoplasmic membrane. This process uses a dedicated secretion apparatus coded by *comAB*. Competence is triggered by CSP stimulation of its receptor comD, a membrane-bound histidine kinase that autophosphorylates and transphosphorylates its cognate response regulator comE. ComE binds to a specific site in the DNA activating the early competence genes, including *comABCDE*, leading to further accumulation of CSP, and *comX*. This latter gene encodes a sigma-X factor that activates the transcription of the late competent genes.

Two recent transcriptomic studies using DNA microarrays have identified over 100 genes induced by CSP. Ninety-one genes were common to both studies and included 17 early *com* genes and 60 late *com* genes. Of significant interest, it was shown that only 22 genes of the *com* regulon are required for transformation, suggesting that the role of competence extends well beyond genetic transformation.

Recently, it was discovered that competent cells have the capacity to trigger the lysis of genetically identical as well as nonidentical noncompetent cells. This phenomenon of 'fratricide' predation has been termed allolysis. A bacteriocin system named CibABC (competence-induced bacteriocin) consisting of a two-peptide CibAB bacteriocin and its immunity factor CibC has been identified. It was shown that CibAB is absolutely required for allolysis but cannot promote lysis *per se*. Gene *cibC* encodes a 65 amino acid peptide with two predicted transmembrane segment. Evidence suggests that cell-to-cell contact is necessary for allolysis to occur. Guiral and colleagues proposed that CibAB may insert into the membrane of noncompetent cells (which lack CibC) and deplete them of energy. Cell lysis of noncompetent cells would then occur as a consequence of the action of three amidases – LytA, LytC, and CbpD.

The observation that allolysis occurs during the transient state of competence (*cibABC*, *lytA*, and *cbpD* are late genes of the *com* regulon) is probably intimately related to its biological significance. Current hypotheses on the contribution of allolysis to the biology of pneumococci have been reviewed recently by Guiral and colleagues. The latter not only include DNA release for genetic transformation but also a role in colonization and/or virulence, competition between strains, and release of virulence factors.

Bacteriophage of Pneumococci

Pneumococccal bacteriophage of the lytic and temperate types were first described in 1970s. Subsequent work on pneumococcal phage found that the lytic enzymes of many of them shared a high identity (at the protein and DNA level) to the major pneumococcal autolysin LytA.

Work by Ramirez and colleagues found that the incidence of prophage among a collection of 791 diverse pneumococcal clinical isolates was high – 76% – and that multiple carriage was frequent. The initial observations were based on the fact that multiple bands of SmaI-digested total DNA separated by PFGE hybridized with a *lytA* probe. Further assays on a subset sample of 17 isolates showed that the majority of the isolates tested (11 of 17) contained functional prophages that entered the lytic cycle following mitomycin induction.

Subsequent studies by the same authors showed that some PFGE subtypes of SmaI-digested total DNA could be explained by changes in the number and chromosomal localization of prophages, suggesting that prophage carriage could be used as a molecular epidemiological marker.

In recent years, the use of pneumococcal phage lytic enzymes as anti-infective agents has been explored. It has been shown that a purified pneumococcal bacteriophage lytic enzyme (Pal) was able to kill pneumococci of common serotypes including highly penicillin-resistant strains in just a few seconds. In an animal model of colonization, pneumococci were eradicated without disturbing the normal flora. Following these initial studies, the therapeutic use of bacteriophage enzymes has been tested with success in different animal models of disease (bacteremia, endocarditis, and otitis media). More recently, the pneumococcal LytA autolysin was found to be a potent therapeutic agent in experimental peritonitis-sepsis caused by highly β-lactam-resistant *S. pneumoniae*.

Genome Sequencing

The first three pneumococcal genomes sequenced were TIGR4 (serotype 4 invasive disease isolate from Norway), R6 (avirulent, noncapsulated derivative of the serotype 2 strain D39), and G54 (serotype 19F derivative of a serotype 15 clone from Spain). All were published in 2001. Since then an increasing number of strains have been sequenced as novel and affordable sequencing technologies and more powerful bioinformatic tools became available, lowering dramatically the costs and time needed for such projects. As of April 2008, genome sequencing information of at least 18 strains is publicly available. Other genome sequencing projects are ongoing. Among the genome sequences available are representatives of the most successful pandemic drug-resistant clones as well as representatives of susceptible strains that are frequent causes of IPD in countries of the developing world. In addition, the capsular operons of the 91 capsular types described to date (including the recently described serotype 6C) have been sequenced.

The Distributed Genome Hypothesis

The size of the pneumococcal genome depends on the strain analyzed. Among the strains sequenced so far it is generally in the range of 2.0–2.2 Mb. Evidence obtained up to now supports the distributed genome hypothesis, which, in short, states that pneumococci (as other highly recombinogenic bacteria) contain a supragenome that is much larger than the genome of any individual strain. Each pneumococcus genome contains a core set of genes (common to all pneumococci) and a noncore set of genes that are part of a large pool of genes.

The largest comparative genomic analyses published so far used the combined analyses of the genome sequences of 17 strains, to estimate the number of orthologous clusters in the pneumococcal supragenome based on the finite-supragenome model. The model predicted that the pneumococcal supragenome should contain approximately 5100 orthologous clusters of which 27% are core, 41% are unique, and the remaining 32% are distributed (i.e., present in more than one strain but not common to all).

Pneumococcal Cell Wall: Composition, Structure and Mechanisms of Replication during Cell Division

The cell wall consists of approximately equal proportions of a peptidoglycan and a teichoic acid of unusual chemical composition, the components of which include galactosamine phosphate, 2,4,6-trideoxy-2,4-diaminohexose, ribitol phosphate, and phosphocholine. A membrane teichoic acid of identical primary structure is also present at the cell surface.

High resolution chemical analysis of the peptidoglycan performed on many clinical isolates demonstrated that the composition of the pneumococcal peptidoglycan was specific for the species. The muropeptide units of the peptidoglycan of pneumococci are either directly crosslinked to one another or are crosslinked via short dipeptide branches consisting of either seryl alanine or alanyl alanine units, which are attached to the free amino group of the stempeptide lysine residues. While direct crosslinks are the dominant mode of crosslinking in penicillin-susceptible pneumococci, the branched peptides become the prominent mode of crosslinking in penicillin-resistant strains.

Genetic determinants of the dipeptide branches have been acquired by pneumococci from an as yet unidentified extra species source. The genetic determinants *murM* and *murN* are essential for the expression of high-level penicillin resistance.

The choline residues of the teichoic acid polymers were shown to perform critical and multiple physiological functions in *S. pneumoniae* and most recent studies also identified choline residues as major modulators of pneumococcal virulence in animal models of pneumococcal disease. The presence of choline residues was shown to be essential for a wide range of physiological phenomena such as separation of daughter cells at the end of cell division (LytB), sensitivity of pneumococci and pneumococcal cell walls to degradation by the pneumococcal autolysin LytA; choline residues were shown to be essential for genetic transformation and the cell wall choline units were shown to serve as noncovalent attachment sites for a group of proteins, the so-called choline-binding proteins, which serve multiple functions in the interaction of pneumococci with their eukaryotic hosts. The choline residues are also sites for the attachment of the C-reactive protein and for the large family of myeloma proteins.

Fine Structure of Cell Wall

The shape and size of microbial cells is imprinted in their cell walls, which – in the case of *S. pneumoniae* – resembles an American ‘football’ with diameters of 1.0–1.5 μm from tip to tip and with a ‘waistline’ of 0.5–0.8 μm. The cell wall of *S. pneumoniae* appears in electron microscopic thin sections as a trilaminar layer directly underneath the somewhat amorphous polysaccharide capsule.

Fine structure studies were done on the ‘rough’ (nonencapsulated) *S. pneumoniae* strain R6, which is a derivative of strain R36A. The overwhelming majority of laboratory studies that have been done on pneumococci over the past several decades all used either R36A, R6, or some simple mutant derivatives of these two strains. R36A was derived from strain D39, a clinical isolate expressing a serotype 2 capsular polysaccharide on its surface. R36A was selected after 36 serial passages of D39 in medium containing anticapsular type II antibody. Strain R36A was shown to carry a 7510 bp deletion involving five of the genetic determinants of the capsular type II genetic locus. The fully sequenced strain R6 is not isogenic with R36A: R6, unlike its ‘parent’ strain, carries an inactivating point mutation in the *dlt* gene and it also has a muropeptide composition in which branched peptides typical of penicillin-resistant isolates are frequent.

Replication during Cell Division

Similar to the case of other streptococci, the pneumococcal surface is inherited in a conservative manner during growth: old hemispheres of the cell surface assembled during the previous cell division are passed on intact to the daughter cells in each subsequent cell generation (**Figure 4**(**a**)).

The various polymeric components of the pneumococcal cell wall – the peptidoglycan, teichoic acid, and capsular polysaccharide chains – all appear to enter the

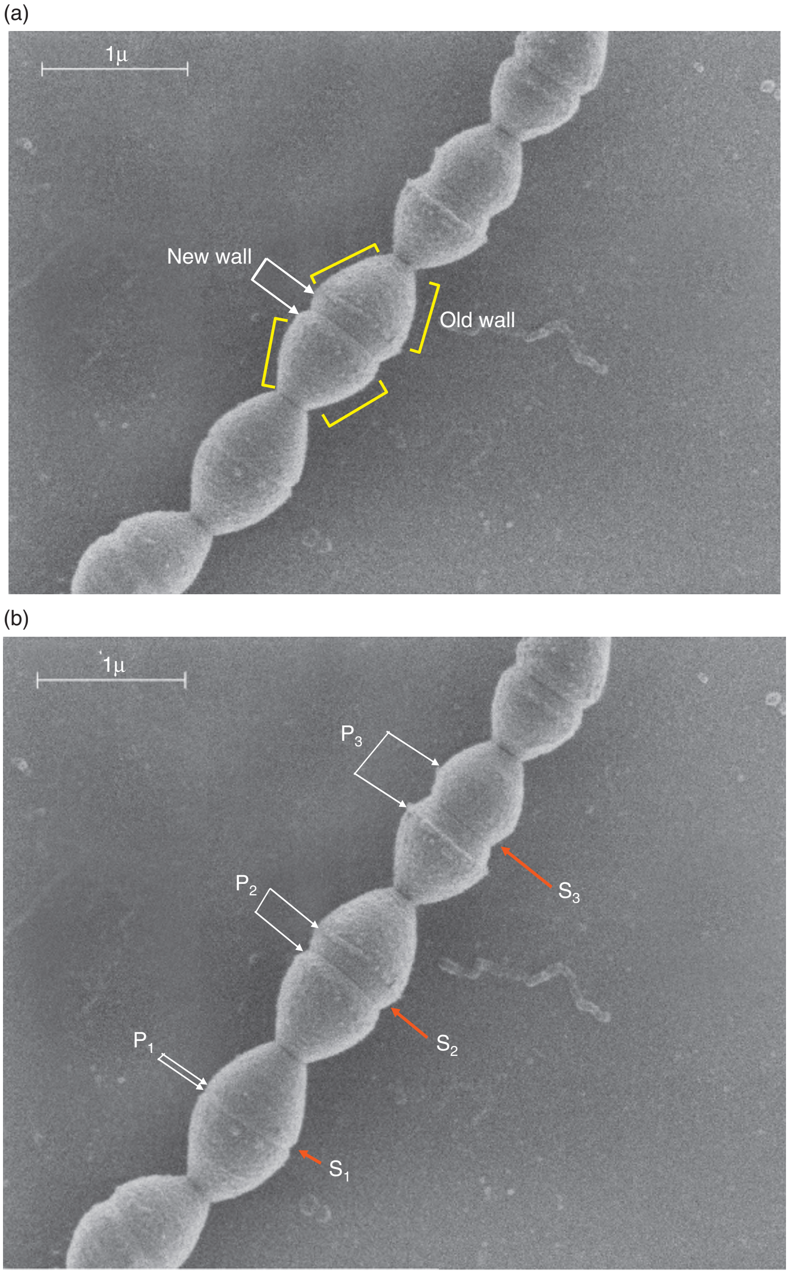


Figure 4 (a) Conservation of ‘old’ hemispheres of the cell wall and insertion of ‘new’ wall material in the vicinity of the new cellular septum. (b) Symmetrical movement of the cell surface during cell division of *S. pneumoniae*. Scanning electron micrograph of a chain-forming pneumococcus showing cells in different stages of cell division. In the cell, at the beginning of a division cycle, the ‘raised cell wall band’ is shown just after separation to two ring-like structures catalyzed by enzymes participating in the peripheral cell wall synthesis (P1). Also shown is the beginning of septal wall synthesis (S1). During subsequent stages the distance between the two ring-like structures associated with peripheral wall synthesis (P2 and P3) increases gradually and so does the advance of the septal wall synthesis ring (S2 and S3).

pneumococcal surface at a single centrally located growth zone. The wall teichoic acid chains are attached by covalent bonds to the peptidoglycan – presumably to the muramic acid C-6 hydroxyl residues, which are also the attachment sites for the capsular polysaccharide chains and the recently discovered *O*-acetyl groups as well. The enzymes catalyzing these three types of reactions competing for the same peptidoglycan attachment sites are not known at the present time; neither is the physiological control of these reactions well understood.

Replication of the cell wall seems to occur through the carefully coordinated functioning of two synthetic systems: one that appears to catalyze a peripheral cell wall synthesis (see P in **Figure 4**(**b**)), and a second one that catalyzes septal growth of the cell wall (see S in **Figure 4**(**b**)). In a newly born cell the future site of cell division is indicated by a 'raised cell wall band' located at the perfect center of the coccoid-shaped cell. The next step in cell division is an apparent splitting of the raised cell wall band into two ring-like structures that proceed to 'move' in a symmetrical fashion to the left and to the right of their initial position. Thus, as cell division progresses, the distance between the two rings increases (see P1, P2, P3 in **Figure 4**(**b**)) In parallel with this movement is the activity of the second – septally located – cell wall synthetic system, which produces cell wall along the newly developing septum (see S1, S2, S3 in **Figure 4**(**b**)).

Recent work by Morlot and colleagues indicates that the three bifunctional PBPs of pneumococci (PBP1a, PBP1b, and PBP2A) as well as the two monofunctional PBPs (PBP2B and PBP2X) are all colocalized to the center of the newly born pneumococcus and seem to remain there during the entire course of cell division. The localization of PBP3, a DD-carboxypeptidase, seems to be unique in that PBP3 is present along the entire pneumococcal surface except the septal zone, suggesting an abundance of donor muropeptides available at the site of active cell wall growth. A model to explain the coordination of septal versus peripheral cell wall growth has been proposed recently. The exact functioning and localization of other cell division-related proteins possibly involved with cell division such as FtsA, the gene products of DivIVA, and the newly described putative murein hydrolase PcsB is not clear at the present time.

PostScript

Writing an 'Encyclopedia' article on the Pneumococcus was a risky undertaking in the era of Pubmed and Google search: Highly specific and detailed information fitting a reader's particular curiosity is instantly available upon pushing a few buttons on the computer. Also, the number of contributors and contributions to the microbiology of *Streptococcus pneumoniae* has increased substantially since the last edition of the Encyclopedia in the year 2000. A Pubmed search of all papers with *S. pneumoniae* in their titles and/or abstracts published between January 2000 and January 2008 identified more than 8000 publications with topics about equally divided between pneumococcal disease, epidemiology, and drug resistance plus a number of papers on clinical trials. Even a comprehensive listing of these was clearly impossible.

With these caveats in mind, we provided the reader with an admittedly subjective sampling of the more recent activities and publications of the field, a kind of updated "subject index with a narrative". Using this treatise the interested reader may then launch his/her more detailed search of the literature.

Acknowledgments

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Relevant Website

www.sph.emory.edu/PMEN/ – Pneumococcal Molecular Epidemiology Network (PMEN)

Stress, Bacterial: General and Specific

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Defining Statement
Introduction
The Stress Response is Two-Pronged
Specific Stress Response
General Stress Response
Regulation of Stress Response
Concluding Remarks
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Glossary

ancillary factors Proteins or other molecules that influence RNA polymerase activity.

antiporter A protein in cytoplasmic membrane that brings about exchange of external protons and a cellular ion/compound.

electrophiles Compounds that accept electrons.

eutrophic environments Environments made nutrient-rich primarily through human activity.

inclusion bodies Precipitated and denatured proteins inside a cell. These are usually formed in bacteria when a heterologous protein is overproduced.

periplasm Space between outer and cytoplasmic membranes in Gram-negative bacteria.

pex proteins The core set of proteins induced in response to diverse stresses.

porins Proteins in the bacterial outer membrane that form water-filled pores, permitting transport.

proteome Complete protein profile of a cell.

redox cycle A reduction reaction that generates unstable radicals. These give their electrons to oxygen generating reactive oxygen species (ROS). The radical is changed back to the original compound and becomes available for further ROS generation.

sigma factors Small proteins that combine with the RNA polymerase core enzyme. The resulting RNA polymerase holoenzyme can transcribe various genes. Each species of RNA polymerase generally recognizes specific promoter sequences.

transcriptome Complete gene transcription profile of a cell.

Abbreviations

cAMP	Cyclic AMP
GSR	general stress response
HARVs	high aspect to ratio vessels
HGH	Human growth hormone
HPK	histidine protein kinase
IHF	integration host factor
LpDH	lipoyldehydrogenase
OMPs	outer-membrane proteins
QH_2	hydroquinone
RNAP	RNA polymerase
ROS	reactive oxygen species
RR	response regulator
rRNA	ribosomal RNA
sRNAs	Small RNA
TIR	translational initiation region
UTR	untranslated region

Defining Statement

Bacteria counter stress at two levels, specific and general, to escape a given stress and to acquire greater robustness. I will discuss here the mechanisms of escape, increased cellular robustness, and the molecular mechanisms that enable a bacterium to shift from rapid growth mode to stasis and enhanced resistance.

Introduction

Bacteria, like other living things, require certain physico-chemical conditions in order to thrive. Usable nutrients need to be sufficiently available, temperature and pH maintained within specific limits, and toxic influences absent. Under such optimal conditions, bacteria grow at maximal rates of which they are genetically capable. The animal gut flora encounters such conditions after the host has taken a meal, intracellular pathogens often immediately after invasion, and environmental bacteria in, for example, eutrophic environments. But such conditions are rare and fleeting, and as a rule, bacteria in nature exist under conditions that are not only suboptimal but can be outright hostile to their survival, exposing them to diverse kinds of stresses.

A common stress is lack of food. Thus, the gut flora by its rapid growth soon exhausts the nutrients passed on to the host intestine and progresses from feast to famine, and the same is likely true of an intracellular pathogen. While eutrophic environments are on the rise due to human activities, much of the natural environment nevertheless remains severely nutrient-poor. Oceans are estimated to have 0.8 mg carbon nutrients per liter, and the concentration of individual carbon compounds in fresh water is often as low as 6–10 $\mu g\,l^{-1}$. Similarly, soils as rule possess little usable nutrients, as most of the 0.8–2.0% carbon in this environment is humus, which bacteria for the most part cannot use. In other natural environments, bacterial growth is restricted by the scarcity of other nutrients, such as nitrogen, phosphorus, and/or iron.

The fluctuating conditions in nature expose bacteria to additional stresses. Diurnal and seasonal changes in temperature can be significant, and a host of abiological and biological factors can result in exposure to a variety of insults, such as pH, osmotic, shear, and oxidative stresses. The pathogenic bacteria have not only to be adept at surviving these stresses during their extra-host existence but also to be able to cope with deleterious influences as they attempt to survive in the host in disease initiation. For example, to infect a host, *Salmonella enterica* serovar Typhimurium, which causes a typhoid-like disease in mice, has to survive passage through the stomach where the average pH over a 24-h period is as low as 1.5. It then invades the interior of the host by infecting the microvilli of the gastrointestinal tract, which are low-shear environments, and it is then ingested by the host macrophage, where additional insults await – oxidative stress, nutrient deprivation, and low pH. To meet such threats to survival, bacteria have evolved elaborate adaptive responses; these are the subject of this article with special emphasis on starvation, although other stresses are also considered.

The Stress Response is Two-Pronged

Bacteria meet the challenge to survival posed by stresses by a two-pronged strategy. One is aimed at neutralizing and escaping the specific stress that is encountered. This response tends to be unique to each stress; thus the proteins a bacterium needs to escape, for instance, oxidative stress are different from those it utilizes to escape starvation. This is termed the specific stress response. The second component of the stress response is aimed at preventing and repairing the damage that the stress might cause and is activated as an insurance policy, since there is no guarantee that the first response will succeed in preventing the deleterious effects of the stress. All stresses, if not neutralized, lead to a common outcome, namely damage to the cell macromolecules, and the second tier of the stress response is aimed at preventing and repairing this damage. Thus, this facet of the stress response results in making bacteria resistant not only to the stress that is experienced but also to others, and is thus termed the general stress response (GSR).

Specific Stress Response

Starvation

The first definitive indication that bacteria respond to stresses by a two-pronged strategy came when the proteomes of bacteria subjected to different stresses were examined. For example, starvation for carbon, nitrogen, or phosphorus resulted in the induction not only of proteins unique to that starvation condition but also to that of a core set of proteins that was common to all the starvation conditions (referred to as Pex proteins). Exposure to stresses mechanistically different from starvation, *viz*., oxidative, osmotic, pH, and others, also led to the induction of unique and common proteins, many of the latter being the same as the core starvation (Pex) proteins. Based on these findings, it was proposed in 1989 that the proteins unique to a specific stress were concerned in enabling the bacteria to neutralize that particular stress, while the core set of proteins was concerned with conferring resistance to stresses in general. This has been found to be the case. In this section, I will discuss the physiological role of selected proteins that are concerned with the escape response; the function of the Pex proteins that confer general resistance is discussed in subsequent sections.

Examples of proteins concerned with escaping stresses are provided in **Table 1**. Starvation-escape response consists in the synthesis by bacteria of enzymes that amplify their capacity to obtain the scarce nutrient. This is accomplished either by increasing the concentration of the relevant enzymes or by synthesizing a new set that possess a higher affinity for the nutrient. Either way, a superior capacity is acquired to scavenge the scarce nutrient. The proteins that are induced can concern every metabolic feature: transport through the outer and cytoplasmic membranes, enzymes involved in substrate capture, and those responsible for subsequent flux through the metabolic pathways. Thus, when phosphate concentration falls below some 1 $mmol\,l^{-1}$ in the environment, cells increase the protein PhoE, which is a porin facilitating the passage of phosphate compounds through the outer membrane

Table 1 Selected escape-response proteins

Protein	*Function*
Phosphorous starvation	
Pst	High-affinity phosphate transport system
PstS (also called PhoS)	Periplasmic Pi-binding protein required for PstS function
PhoE	Porin that facilitates Pi transport through the outer membrane
PsiB and PsiC	Glycerol phosphate transport systems
Bacterial alkaline phosphatase	Carbon–phosphorus bond lyase
Carbon starvation	
Periplasmic-binding proteins (e.g., MalE)	Enhanced transport (e.g., maltose)
Glucokinase	Substrate capture (glucose)
Lactate dehydrogenase	Substrate capture (lactate)
$\widetilde{\beta}$-Galactosidase	Substrate capture (lactose)/metabolic potential amplification
CstA	Substrate capture (peptides)/metabolic potential amplification
Glycerol kinase	Substrate capture (glycerol)
Glucose-6-phosphate dehydrogenase	Enhanced flux through catabolic pathways
Phosphofructokinase	Enhanced flux through catabolic pathways
Pyruvate kinase	Enhanced flux through catabolic pathways
Aconitase	Enhanced flux through catabolic pathways
Isocitrate dehydrogenase	Enhanced flux through catabolic pathways
Malate dehydrogenase	Enhanced flux through catabolic pathways
Other stresses[a]	
Aerobactin (iron starvation)	Iron chelator
Glutamine synthetase (nitrogen starvation)	Substrate capture
Kdp (potassium starvation)	High affinity K^+ transport
Superoxide dismutase (oxidative stress)	Decomposes superoxide
KatE (oxidative stress)	Catalase
KatG (oxidative stress)	Catalase
Thiol peroxidase (oxidative stress)	Thiol-dependent hydroperoxidase
Sulfate adenylyltransferase (oxidative stress)	Cysteine biosynthesis
Cysteine synthase (oxidative stress)	Cysteine biosynthesis
ChrR (oxidative stress)	H_2O_2 quencher
Lysine decarboxylase (acid stress)	Generates cadaverine that buffers the cytoplasm
CadB (acid stress)	Brings about exchanges of cellular cadaverine for medium lysine
UreI (acid stress)	Increases membrane permeability to urea which, through urease activity, buffers the cytoplasm

[a] Text in parentheses indicates the stress.

into the periplasmic space of *Escherichia coli*. Here, it interacts with a high affinity-binding protein (PstS), also induced under these conditions, promoting efficient functioning of PhoE. The compounds thus transported to the periplasm are hydrolyzed by another protein induced by phosphate starvation, the bacterial alkaline phosphatase, generating Pi. Rapid transport of the latter across the cytoplasmic membrane is ensured by the fact that a high affinity Pi transport system, Pst (energized by ATP; K_m for Pi, 0.16 μmol l^{-1}), is concomitantly induced under these conditions, replacing the low affinity Pit system (energized by proton motive force; K_m for Pi, 25 μmol l^{-1}) that operates under phosphate-sufficient conditions.

This pattern has been demonstrated in several bacteria also when limitation for other nutrients is encountered. Carbon-scarce cells often also synthesize high affinity-binding proteins, for example, MalE, which binds maltose facilitating its transport into the cell. When *Pseudomonas* or enteric bacteria utilizing lactate or glucose as carbon source were subjected to the limitation of these substrates, they greatly increased the synthesis of lactate dehydrogenase or glucokinase, respectively. Concomitantly, there was a marked induction of several enzymes of glycolysis and tricarboxylic acid cycle, ensuring effective channeling of low levels of catabolites through them. Large amounts of glutamine synthetase, which catalyzes the first step in ammonium assimilation, are synthesized during ammonium limitation, and induction of high affinity substrate-capturing proteins occurs also during potassium and glycerol scarcity. In the former case, the cells shift to the Kdp system (high affinity; energized by ATP) from the Trk transport system (low affinity; energized by proton motive force) that is used when potassium is plentiful. Cells grown on nonlimiting concentrations of glycerol utilize a low affinity pathway for its catabolism whose initial step is catalyzed by glycerol dehydrogenase; under glycerol scarcity on the contrary, a high affinity pathway initiating with glycerol kinase is utilized.

Iron-challenged cells increase the synthesis of the iron siderophore, aerobactin. Thus, a combination of the synthesis of high affinity transport and other proteins coupled with a general increase in the level of metabolic enzymes ensures that the cells can effectively scavenge and utilize the scarce nutrient from the environment.

These measures can of course not always succeed in alleviating starvation. For instance, cells growing on glucose can synthesize any amount of enzymes to facilitate its utilization, but this would not help if this substrate becomes completely absent from the environment. An additional measure is therefore employed, which is to derepress the synthesis of enzymes for substrates other than glucose counting on the chance that the constantly fluctuating conditions might promote their appearance in the environment. Thus, cells subjected, for instance, to glucose starvation also synthesize enzymes such as β-galactosidase and CstA, which confer on them the capacity to utilize lactose and peptides, respectively, thereby acquiring the capacity to cast a wider net for alleviating carbon starvation.

Oxidative Stress

Ground state oxygen has two unpaired spins, and the constraints of quantum mechanics, and the resulting spin restriction, hinder its divalent reduction. This favors the univalent pathway that generates highly reactive (and toxic) oxygen species (ROS). Consequently, oxidative stress from ROS is a constant threat to bacteria and other living entities. Bacterial respiratory chains (like those of the mitochondria) leak ROS. Phagocytes possess a membrane-bound NADPH reductase, whose function is to catalyze one-electron reduction of O_2 to generate ROS so as to kill the invading bacteria. When plant cells come in contact with soil-dwelling bacteria, such as *Pseudomonas putida*, they release an immediate burst of H_2O_2. Many electrophiles generated internally by bacteria or those found in the environment are also a source of oxidative stress. Examples are quinones, nitro-compounds, chromate, and several dyes; quinones such as plumagin and juglone are secreted by plants as defense mechanisms against bacteria. These compounds are vicariously attacked by cellular metabolic enzymes such as glutathione and cytochrome *c* reductases, and lipoyl dehydrogenase (LpDH), which reduce them by one-electron transfer. The result is the generation of reactive radicals, such as semiquinones and Cr(V), which set up a redox cycle. In this process, the radical (e.g., semiquinone) transfers its electron to O_2 or, depending on the conditions to another molecule (e.g., NO_3), regenerating quinone and producing ROS or other equally destructive oxidizing agents (e.g., nitrosative radicals). With the continued activity of one-electron reducers, the quinone (or other such electrophiles) shuttles back and forth between its quinone and semiquinone valence states, producing large quantities of ROS. These compounds are referred to from here on as 'univalent reduction-prone' electrophiles.

That bacteria do indeed experience severe oxidative stress when exposed to univalent reduction-prone compounds was demonstrated by the use of the intracellular oxidative stress sensor 2′, 7′-dihydrodichlorofluorescein (H_2DCFDA), which is taken up by the cells and emits green fluorescence in the presence of ROS. For instance, *E. coli* cells exposed to chromate do indeed emit green fluorescence (**Figure 1**). Proteome analysis showed that these cells induced several proteins concerned with combating oxidative stress, for example, superoxide dismutase, which decomposes the superoxide radical, and those concerned with cysteine and thiol biosynthesis, which are ROS quenchers. Mutants unable to synthesize these proteins proved more sensitive to chromate killing, and strains with bolstered capacity to synthesize antioxidant defense proteins (such as ChrR; **Table 1**; see below) less so compared to the wild type. Other examples of proteins that permit escape from oxidative stress are given in **Table 1**.

A new class of enzymes, termed ChrR, has recently been discovered, which has a broad range of activity to combat oxidative stress. These enzymes bring about a simultaneous two-electron reduction of univalent reduction-prone electrophiles. Thus, for example, they convert in one step quinone into fully reduced and stable hydroquinone (QH_2), bypassing semiquinone formation. The experimental approach to determine if an enzyme reduces the univalent reduction-prone electrophiles by one- or two-electron

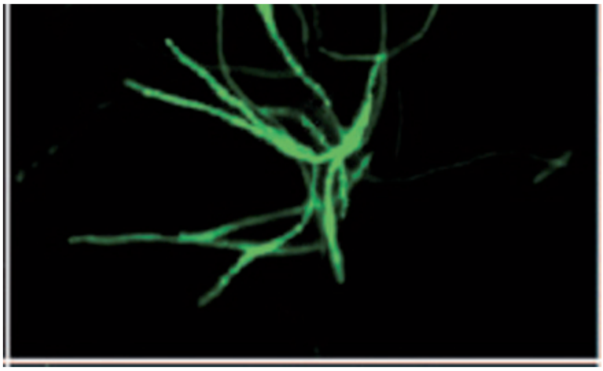

Figure 1 *Escherichia coli* cells exposed to 250 μmol l^{-1} chromate and treated with intracellular ROS sensor 2′, 7′-dihydrodichlorofluorescein. Cells were examined at ×1000 magnification with an Olympus BX60 upright fluorescence microscope. Note that the cells form snakes and fluoresce green; both are indicative of oxidative stress. Reproduced from Ackerley DF, Barak Y, Lynch SV, *et al*. (2006) Effect of chromate stress on *Escherichia coli* K12. *Journal of Bacteriology* 188: 3371–3381.

pathway utilizes pure proteins and a source of electrons, namely NADH or NADPH. It takes advantage of the fact that cytochrome *c* is reduced by semiquinones but not by hydroquinones, and since reduced cytochrome *c* absorbs light of 550 nm wavelength, its reduction can easily be monitored in a spectrophotometer, serving as a facile probe for semiquinone formation. It was found that when quinone was reduced by a number of different cellular enzymes, such as LpDH, large amounts of reduced cytochrome *c* were generated, indicating that the quinone was reduced by one-electron transfer and generated semiquinone. However, when the reduction was catalyzed by the enzyme ChrR, no reduction of the cytochrome was seen (**Figure 2(a)**). Thus, the latter enzyme bypassed semiquinone formation resulting in direct conversion of the quinone to QH_2.

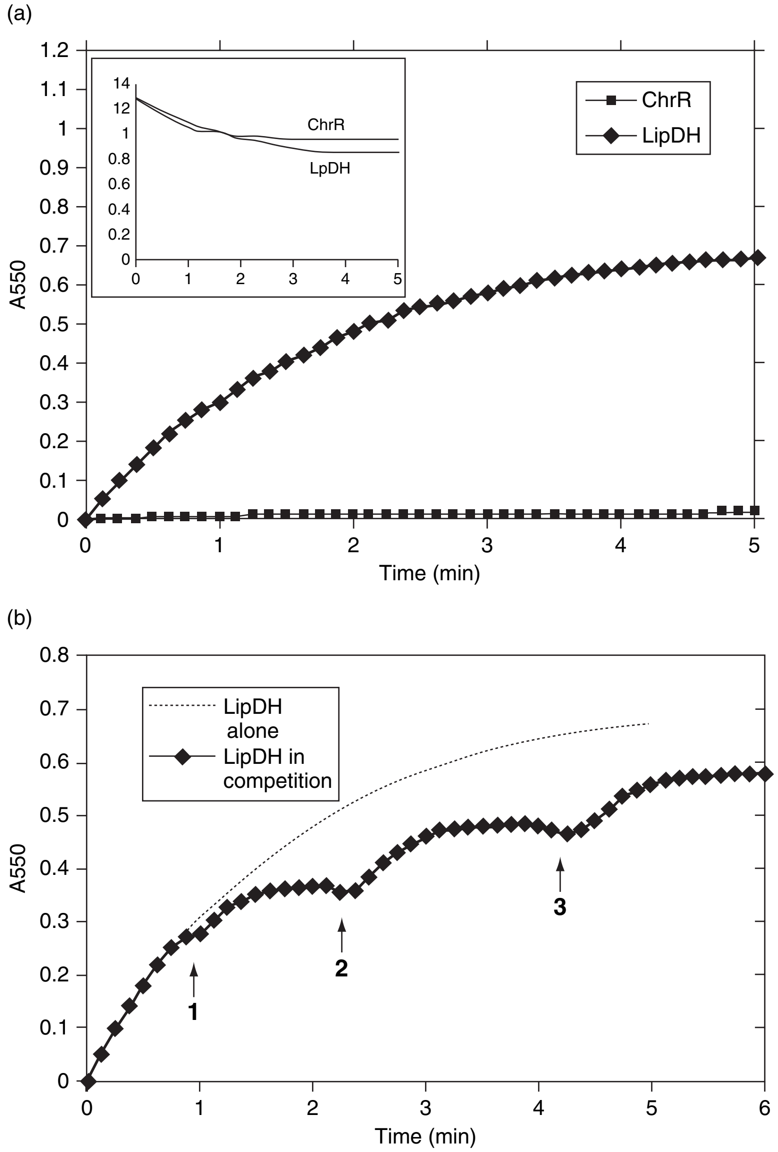

Figure 2 (a) Reduction of cytochrome *c* monitored spectrophotometrically at 550 nm during LpDH- or ChrR-catalyzed reduction of 50 μmol l^{-1} of a quinone species, benzoquinone. The appearance of reduced cytochrome *c* during the LpDH-catalyzed reaction indicates one electron transfer and generation of semiquinone, whereas the lack of this species in the ChrR-catalyzed reaction signifies a divalent mode of quinone reduction that generates QH_2 bypassing semiquinone generation. (b) Addition of ChrR to an LpDH-catalyzed reduction of limiting benzoquinone, at the point marked by *arrow* 1, rapidly arrested the reduction of cytochrome *c* relative to LpDH alone (dashed line). The addition of fresh benzoquinone (arrows 2 and 3) reinitiated cytochrome *c* reduction, but with ChrR now present, only little semiquinone is generated as indicated by very limited cytochrome *c* reduction. This indicates that the presence of the two-electron reducer, ChrR, preempts quinone reduction by the one-electron reducer, LpDH. Reproduced from Gonzalez CF, Ackerley DF, Lynch SV, *et al*. (2005) ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H_2O_2. *Journal of Biological Chemistry* 280: 22590–22595.

In an extension of this experimental approach, limiting concentrations of quinone were used, which ensured that the reaction ceased because all the available quinone in the reaction mix was exhausted. **Figure 2(b)** shows that in such a situation when ChrR is added to an in-progress LpDH-catalyzed quinone reduction, cytochrome reduction is swiftly halted, indicating that the LpDH is no longer generating semiquinone. Addition of further quinone to the reaction mix reinitiated cytochrome *c* reduction but at a very low rate and this too was soon halted. The experiment thus indicated that when ChrR is present, quinone is made largely nonavailable to LpDH, so semiquinone formation ceases. Experiments using other single-electron reducing enzymes have given similar results. Thus, not only ChrR constitutes a safe pathway for the univalent reduction-prone electrophiles, such as quinones, it is also effective in preempting their reduction by the one-electron reducers, thereby affording a two-way protection to the cell exposed to such electrophiles.

There is in fact another level at which ChrR protects the cell against oxidative stress and that is by virtue of the fact that QH_2, which it generates, is an effective quencher of ROS, such as H_2O_2. Strains of *P. putida* devoid of ChrR and those overproducing this enzyme were grown in the presence of $3\,\mathrm{mmol\,l^{-1}}$ H_2O_2. The different cell cultures exhibited lag phases of varying duration, following which normal growth was seen (**Figure 3**). The ChrR overproducing strain was the first to recover, followed by the wild type, and finally the ChrR mutant. The recovery correlated with the ability of each strain to remove H_2O_2 from the medium, indicating that the cellular ChrR bolsters this capacity. Protein carbonylation, which is an indication of oxidative damage, was greatest in the strain devoid of ChrR and least in the one overproducing this enzyme.

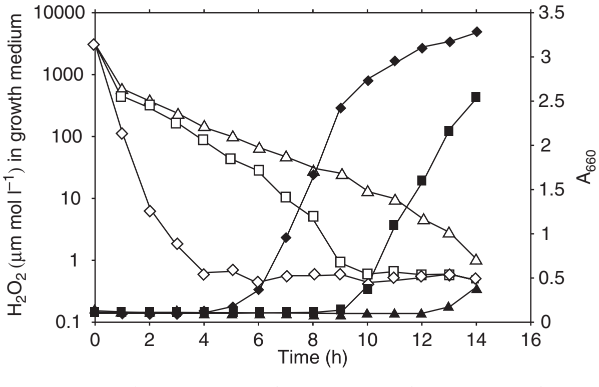

Figure 3 H_2O_2 scavenging (open symbols) and growth (as measured by increase in absorbance at 660 nm, solid symbols) of ChrR-overproducing (◆), wild-type (■), and ChrR-deficient (▲) strains of *P. putida*. Note that the overproducing strain is most efficient in decomposing H_2O_2. Reproduced from Gonzalez CF, Ackerley DF, Lynch SV, *et al*. (2005) ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H_2O_2. *Journal of Biological Chemistry* 280: 22590–22595.

Acid Stress

Escape from acid stress involves a combination of physicochemical approaches as well as the use of special enzymes to ensure that the cytoplasm is not acidified. The former mechanisms include making the cytoplasmic electric potential ($\Delta\psi$) positive, so as to oppose the entry of protons that, of course, are positively charged. It also includes changes in the composition of the cytoplasmic membrane so as to render it less permeant to protons. In *Clostridium acetobutylicum*, for example, exposure to low pH results in a decrease in the ratio of unsaturated to saturated fatty acids and an increase in cyclopropane fatty acid content. An increase in phospholipids with amino acid head groups is another measure that appears to be aimed at decreasing proton permeability of the cytoplasmic membrane.

The enzymes involved are amino acid decarboxylases. A well-studied system involves lysine decarboxylation, which removes CO_2 from lysine and generates cadaverine. Cadaverine picks up a proton, thereby contributing to the deacidification of the cytoplasm. The protonated cadaverine is exchanged for external lysine by the antiporter CadB. Another enzyme involved in the buffering to the cytoplasm is urease, which is thought to be critically important in the ability of the gastric ulcer/carcinoma-causing bacterium *Helicobacter pylori* to colonize the stomach. This bacterium synthesizes a special membrane protein called UreI that enhances urea transport into the cell. Urea is present in the gastric juice, but its permeation into the cell without UreI is too slow to be effective in enabling *H. pylori* to keep a neutral cytoplasm.

General Stress Response

Cross-Protection

As mentioned above, cells respond to different insults not only by measures aimed at escaping a particular stress, but also by bolstering the cellular machinery meant to prevent and repair damage to macromolecules that may result if the escape response fails. The evolutionary basis for this is obvious: the external environment is often so unforgiving that the escape response strategies can often at best have only a partial success and survival necessitates that measures be activated to deal with the damaging effect of stresses. This is the function of the (Pex) core set of proteins that are synthesized regardless of the nature of stress, and they confer on the cell a robustness enabling it to withstand stresses in general.

Proteome analysis of cultures starved for glucose or other nutrients showed that the proteins synthesized fall into different temporal classes and that this synthesis program is essentially complete in 4 h after the onset of starvation. The Pex proteins for the most part exhibit a sustained pattern of synthesis through this period, leveling off at its end. Consistent with their role in enhancing cellular robustness, it was found that inhibition of protein synthesis in a starving culture had a time-dependent effect on starvation survival, with maximum resistance developing after 4 h of protein synthesis during starvation. That the core proteins are involved in conferring general resistance on the cell is further indicated by the fact that the cross-protection that starvation confers on cells against unrelated stresses, for example, heat, oxidation, hyperosmosis, and others (**Table 2**), is also dependent on the time, up to 4 h, for which they have been starved. This phenomenon is illustrated in **Figure 4**(**a**) for the starvation-mediated cross-protection against heat, involving exposure to the normally lethal temperature of 57 °C. For the first 4 h after the onset of starvation, increasing resistance to heat is exhibited the longer the cells are starved, with maximal resistance being acquired within this period. The phenomenon is completely dependent on protein synthesis during starvation, since its inhibition by inclusion in the starvation regime of chloramphenicol or by other means prevents resistance development.

Table 2 Stress-induced resistances

Starvation
Heat
Cold
pH extremes
Oxidation
Hyperosmosis
Cl_2
ClO_2
Ethanol
Acetone
Deoxycholate
Toluene
Irradiation
Antibiotics and other antimicrobials

Reproduced from Matin A (2001). Stress response in bacteria. In: Bolton S (ed.) *Encyclopedia of Environmental Microbiology*, vol. 6, pp. 3034–3046. New York: John Wiley and Sons.

Since the core protein set is synthesized regardless of the nature of stress, it follows that exposure to any stress and not just starvation should confer general resistance. This is indeed the case as is illustrated in **Figure 4**(**b**), which shows that cells exposed to adaptive doses of a variety of mechanistically unrelated stresses become more resistant to lethal concentrations of H_2O_2.

Biochemical Basis

The comprehensive resistance that stresses confer on cells is due to the fact that the core set of proteins are concerned with protecting vital cell macromolecules – proteins, DNA, cell envelope – from damage as well as to bring about repair of any damage that may still result. Envelope protection and reinforcement is afforded by proteins such as D-alanine carboxypeptidase, which increases peptidoglycan cross-linkage, and the products of the *otsBA* (*pexA*) genes which protect the cell membrane by promoting trehalose biosynthesis. Furthermore, several periplasmic proteins concerned with the proper folding of proteins in this cell compartment are upregulated by stress; these

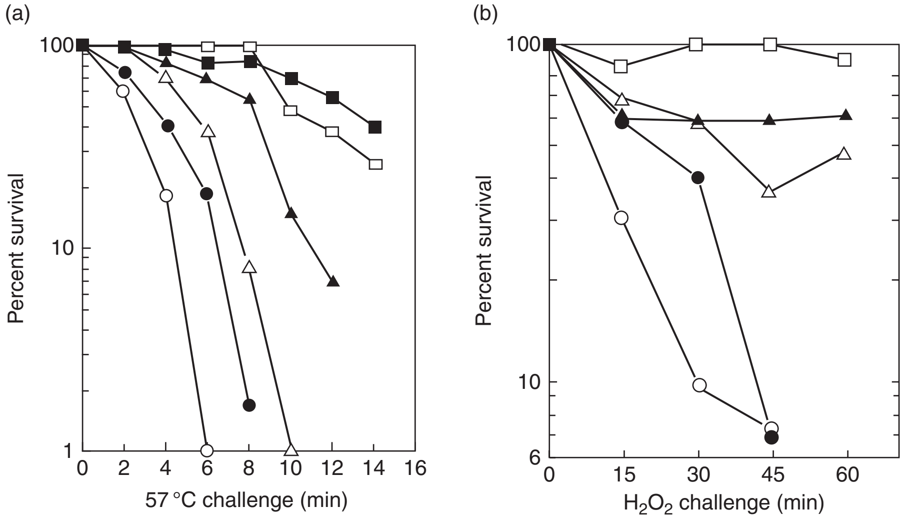

Figure 4 (a) Induction of thermal resistance in *Escherichia coli*. Cells grown at 37 °C were exposed to 57 °C during exponential growth (o), or at 1 h (△), 2 h (▲), 4 h (□), or 24 h (■) after glucose exhaustion from the medium. (•) Represents culture starved in the presence of chloramphenicol. (b) Comparison of the H_2O_2 resistance of glucose-starved *E. coli* cultures to growing cultures adapted by heat, H_2O_2, or ethanol. Symbols: (o) untreated; (•) ethanol-adapted; (△) heat-adapted; (▲) H_2O_2-adapted; (□) glucose-starved. Reproduced from Jenkins DE, Schultz JE, and Matin A (1988) Starvation-induced cross protection against heat or H_2O_2 peroxide challenge in *Escherichia coli*. *Journal of Bacteriology* 170: 3910–3914.

include Dsb proteins that play a role in the formation or isomerization of disulfide bonds in proteins secreted into the periplasm, and peptidyl-prolyl isomerases concerned with the proper folding of proline-containing substrates. A consequence of stress is the accumulation in the periplasm of misfolded outer-membrane proteins (OMPs) due to the stress and excessive OMP synthesis. The OMP mRNAs are unusually stable. Two small noncoding RNAs, RybB and MicA, are induced under stress, especially the envelope stress, which selectively accelerates the decay of these mRNAs, thereby minimizing stress-induced damage by preventing excessive OMP production.

Protein repair

This is brought about by proteins called chaperones, which are a large and diverse group with indispensable physiological roles under all growth conditions, but which become more important under stress. Apart from conferring stress resistance, the chaperones are responsible for proper folding of nascent proteins and protein translocation across membranes. The chaperones DnaK, DnaJ, and GrpE, as well as GroEL and GroES are among the most extensively studied. These proteins are widely conserved through evolution: hsp70 is the eukaryotic homologue of the bacterial chaperone DnaK and hsp60 that of GroEL.

It is thought that the nascent polypeptide chains or denatured proteins (referred to from here on as 'substrate proteins') bind DnaK and DnaJ (**Figure 5**). Interaction between the chaperones in the presence of ATP results in the formation of a ternary complex consisting of the substrate protein, DnaK–ADP, and DnaJ. Dissociation of this complex is mediated by interaction with GrpE and by binding of ATP. The final stages of folding/repair in most cases involve GroEL and GroES. This model is supported by several lines of evidence. For example, the denatured enzyme rhodanese aggregates in a buffer solution, but not in the presence of DnaK, DnaJ, and ATP, as the protein is protected by the ternary complex formation. Addition of GrpE, GroEL, and GroES results in efficient refolding and activation of the enzyme. In bacteria lacking these chaperones, newly synthesized proteins aggregate

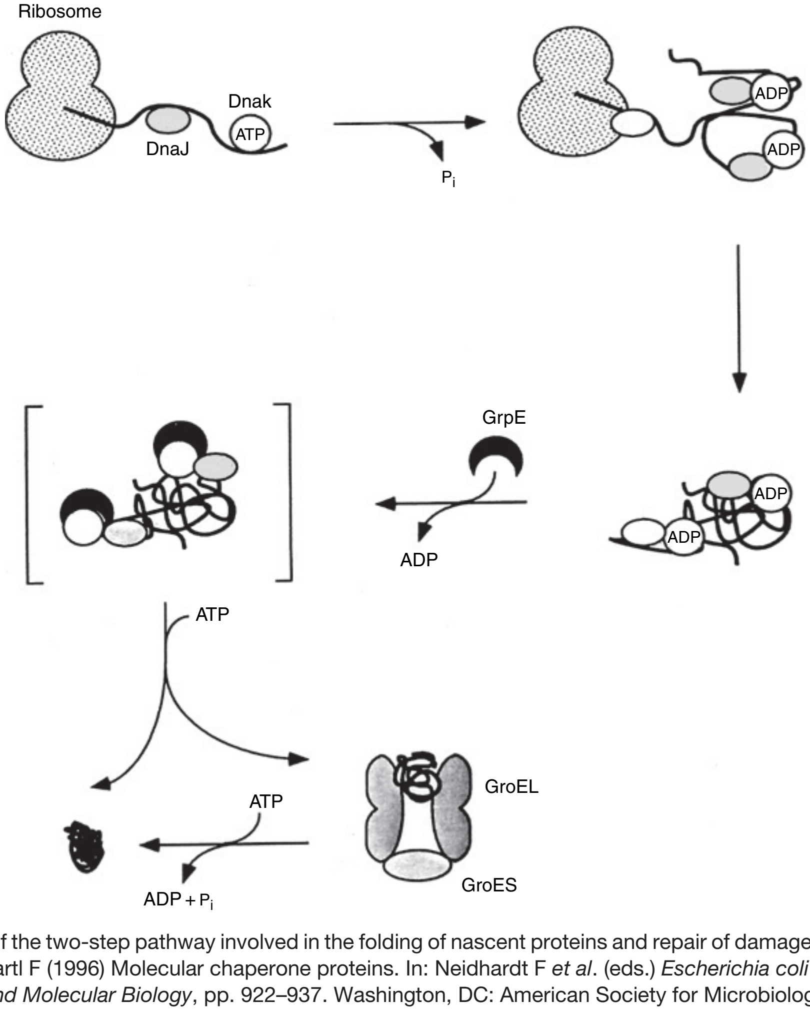

Figure 5 Schematic of the two-step pathway involved in the folding of nascent proteins and repair of damaged proteins. Reproduced from Mayhew M and Hartl F (1996) Molecular chaperone proteins. In: Neidhardt F *et al*. (eds.) *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 922–937. Washington, DC: American Society for Microbiology.

in vivo. However, this aggregation is prevented if the chaperone production is restored. Similarly, proteins imported into the yeast mitochondria from the cytosol show defective assembly in mutants missing hsp60 (GroEL homologue), and most soluble denatured proteins of *E. coli* form complexes with GroEL as a prelude to their repair. Strikingly, proteins in their native state do not interact with the chaperones. Exposure to stresses results in association of a large number of proteins *in vivo* with chaperones presumably to escape damage. In essence, chaperones are slow ATPases, which, when bound to ADP, have a high affinity for denatured proteins, but a low affinity for them when bound to ATP. These characteristics determine the duration of their action on an unfolded part of a protein and ensure the continuation of the process until renaturation is complete.

Bacteria are often used in industry and laboratory to overproduce heterologous proteins as the process is fast and economical. However, often the overproduced protein is denatured within the cell and precipitates, resulting in the formation of inclusion bodies. A protective role against this denaturation for DnaK was demonstrated by its overproduction in the cells. Human growth hormone (HGH) is produced industrially using *E. coli* transformed with a high copy number plasmid containing the *hgh* gene that encodes this hormone. In control cells producing normal levels of DnaK, the HGH produced in the cell formed massive inclusion bodies, but in cells overproducing this chaperone there was marked breakup of these bodies (**Figure 6**) and a corresponding increase in the soluble hormone.

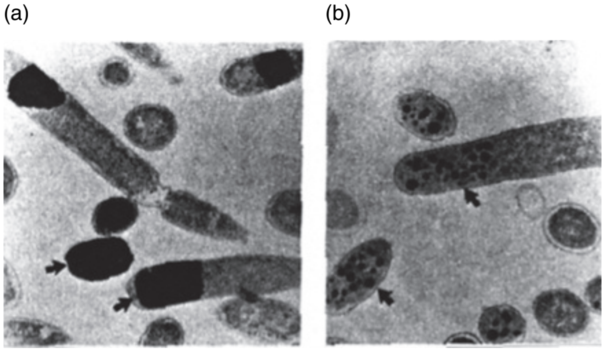

Figure 6 Transmission electron micrographs of *Escherichia coli* cells fixed in late exponential phase growth from cultures overproducing HGH protein. (a) Overproduction of HGH alone; (b) HGH overproduction along with that of DnaK. Note that in the latter, the HGH inclusion bodies are much smaller; there is corresponding increase in soluble HGH. Magnification, 26 000 ×. Reproduced from Blum P, Velligan M, Lin N, *et al*. (1992) DnaK-mediated alterations in human growth hormone protein inclusion bodies. *Biotechnology* 10: 301–303.

DNA repair

Several enzymes induced by stresses are concerned with DNA repair. Examples are endonuclease III and IV, Dps (PexB), and AidB, which reverse DNA methylation. A role for DnaK in DNA repair has also been reported. A major mechanism for DNA repair is the SOS response, which is activated by many different stresses, such as starvation, oxidative stress, irradiation, and antibiotic treatment, which result in DNA damage. This response promotes various kinds of DNA repair such as excision repair. This is aimed at excising pyrimidine dimers and other bulky lesions found in damaged DNA. The enzymes involved are UvrABC endonuclease, which is made up of proteins encoded by the *uvrA, uvrB*, and *uvrC* genes, helicase II (encoded by *uvrD* gene), DNA polymerase I, and DNA ligase. The UvrABC endonuclease makes incisions on each side of the lesion, generating a 12 to 13 base pair oligonucleotide. Different components of the enzyme act separately in this process. UvrA and UvrB interact to form a UvrA$_2$UvrB complex, which identifies the DNA lesion and locally unwinds it, producing a kink in the DNA of 130°. This is followed by dissociation of the UvrA protein and formation of a stable UvrB–DNA complex, which is acted upon by UvrC to make the incision. The function of helicase II is to release the oligonucleotide and to free UvrC after the excision of the nucleotide. The gap generated by the incision is filled by DNA polymerase I, which carries out the repair synthesis, and DNA ligase, which fills the remaining nick.

Regulation of Stress Response

Shift in the cellular gene expression and protein synthesis profile under stressful conditions involves several factors, *viz*., changes in the concentration of sigma factors, ancillary regulatory molecules, and chemical alteration in certain proteins. Salient examples of each will be discussed.

Sigma Factors

Sigma (σ) factors are small proteins that associate with the RNA polymerase (RNAP) 'core' enzyme and determine what promoter the resulting 'holoenzyme' will recognize (**Figure 7**). The core RNAP (abbreviated as E) is made up of four polypeptides, $\alpha_2\beta\beta'$. Examples of sigma factors that play a role in stress response are σ^{70}, σ^{s}, σ^{32}, and σ^{54}; their holoenzymes recognize specific DNA sequences present in a region called the promoter that is located, as a rule, 10 and 35 nucleotides upstream of the transcriptional start site. The σ^{70} holoenzyme Eσ^{70} is indispensable under all growth conditions and is referred to as the vegetative sigma factor. The consensus promoter sequences recognized by three of these holoenzymes

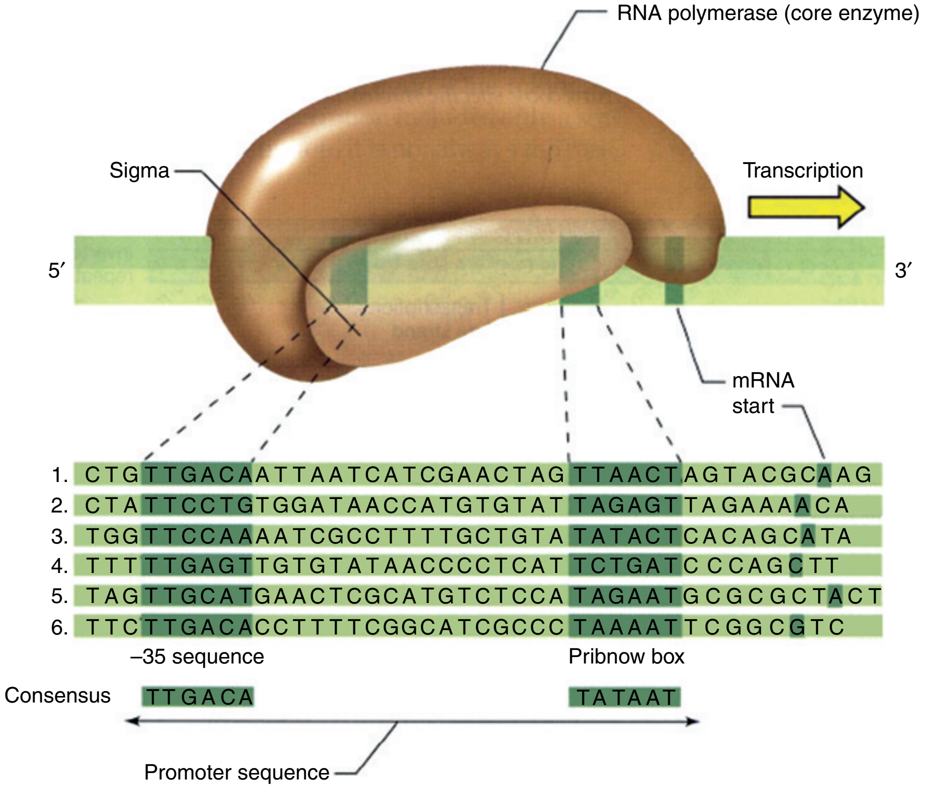
RNA polymerase (core enzyme)
Sigma
Transcription
5′
3′
mRNA
start
1. CTGTTGACAATTAATCATCGAACTAGTTAACTAGTACGCAAG
2. CTATTCCTGTGGATAACCATGTGTATTAGAGTTAGAAAACA
3. TGGTTCCAAAATCGCCTTTTGCTGTATATACTCACAGCATA
4. TTTTTGAGTTGTGTATAACCCCTCATTCTGATCCCAGCTT
5. TAGTTGCATGAACTCGCATGTCTCCATAGAATGCGCGCTACT
6. TTCTTGACACCTTTTCGGCATCGCCCTAAAATTCGGCGTC
–35 sequence
Pribnow box
Consensus
TTGACA
TATAAT
Promoter sequence

Figure 7 Schematic representation of RNA polymerase holoenzyme showing the 2.4 and 4.2 regions, which recognize respectively the –10 and –35 promoter elements. Reproduced from Madigan MT and Martinko JM (2006) *Brock Biology of Microorganisms*, p. vi. Upper Saddle River, NJ: Prentice Hall.

are Eσ^{70}: –10: TATAAT, –35: TTGACA; Eσ^{32}: –10: CATNTA, –35: CTTGAA; and Eσ^{54}: GG-N$_{10}$GC. (Eσ^{s}-recognized promoters are discussed below.) It should be noted that considerable variations from these sequences are tolerated by different species of RNAP, the enzyme species differ in their promiscuity in this respect, and a given promoter sequence can be recognized by different RNAP depending on specific conditions. For example, during starvation or osmotic stress, the transcription of the gene encoding an oxidative stress protection protein, Dps (also known as PexB), depends upon increased cellular levels of Eσ^{s}. However, under oxidative stress, Eσ^{70} with the help of the ancillary factor, called the integration host factor (IHF), allows transcription of *pexB* without Eσ^{s}. Other genes are also transcribed by different RNAP species depending upon the presence of modifying conditions.

While all of these holoenzymes have a role in different stresses, their major role is concentrated on particular conditions. Thus, Eσ^{70} primarily transcribes the exponential phase genes and those concerned with the stress-escape response; Eσ^{32}, the heat shock and starvation genes; Eσ^{s}, the genes that are commonly expressed under stresses in general; and Eσ^{54}, genes of diverse functions including those involved in starvation, flagellar synthesis, and in cell growth on nonpreferred substrates, such as environmental pollutants.

The RNAP holoenzyme most important in inducing the GSR in bacteria is Eσ^{s}, as it controls the expression of some 140 core stress genes that are induced by diverse stresses and are responsible for this response. σ^{s} bears close homology with σ^{70} in critical regions of the sigma protein referred to as regions 2.4 and 4.2, which recognize respectively the –10 and –35 promoter elements. Indeed, Eσ^{70} and Eσ^{s} recognize many of the same promoters *in vitro*. *In vivo* however, under stresses such as starvation, despite the fact that σ^{70} is more abundant in the cells than σ^{s}, Eσ^{s} specifically targets the stress genes. Subtle differences in the promoter sequences and the role of ancillary factors account for this specificity.

Specific features of σ^{s}-recognized promoters

Eσ^{s}-recognized promoters differ from those that Eσ^{70} recognizes in following respects. (1) They possess special features around their –10 region. Thus, a cytosine (C) at –13 position (i.e., 13 nucleotides upstream of the transcriptional start site) and a thymidine (T) at –14 facilitate Eσ^{s} binding to the promoter. Indeed, the –13 C may antagonize Eσ^{70} binding due to the differences in charged amino acids in the two sigma factors. In one instance, introduction of C at this position in a Eσ70 promoter improved its recognition by Eσ^s. Adenine (A)/T-rich stretch is also involved, TAA at positions –6 to –4 being a common feature of Eσ^s-recognized promoters; this feature may allow easier promoter melting (i.e., unwinding of the DNA strands to permit transcription). (2) Eσ^s can tolerate much wider deviations from consensus promoter sequences than Eσ^{70} and can, for example, recognize promoters with degenerate –35 sequences, possibly because it does not need such a sequence *in vivo*, or is able to recognize other sequences in place of this sequence. (3) While the requirement of a 17 base pair space between the –10 and –35 region is a strong preference of Eσ^{70}, Eσ^s is more relaxed in this requirement. Indeed, many Eσ^s-recognized promoters exhibit –35 like elements at other locations. (4) Certain AT-rich sequences present upstream of the –35 region favor Eσ^s binding to the promoter; the C-terminal domains of the RNAP α subunit play a role in this. (5) Both Eσ^{70}- and Eσ^s-recognized promoters tend to possess –10-like elements downstream of the transcriptional start site. Since early transcript complexes retain the sigma factors, these sequences cause the transcription to pause. σ^s is released more rapidly than σ^{70} from these complexes; thus the pause is shorter when Eσ^s is the transcriber, and this may facilitate Eσ^s-mediated transcription of promoters that are recognized by both Eσ^{70} and Eσ^s.

Other factors involved in favoring Eσ^s-mediated transcription

Several *trans*-acting proteins seem to favor Eσ^s-mediated transcription over that of Eσ^{70}. Examples are H-NS, IHF, and Lrp. The mechanisms are not understood. In the case of H-NS, one possible mechanism is that the binding of this protein to a promoter interacting with Eσ^{70}, but not Eσ^s, renders the promoter unavailable for transcription. Changes in core RNAP, cytoplasmic ionic composition, as well as DNA supercoiling can also influence what RNAP species will transcribe a given gene.

A major factor responsible for a shift to different RNAP species under stress is competition for the RNAP core enzyme. The core RNAP concentration in bacterial cell is limiting and different sigma factors have to compete for it. σ^{70} possesses highest affinity for the core enzyme of all sigma factors and is present in excess; this accounts for the predominance of Eσ^{70} in unstressed cells. In stressed cells, even though σ^{70} retains its quantitative dominance, the balance shifts to RNAP species containing the alternate sigmas. Several factors account for this. Eσ^{70} dissociates so that core RNAP concentration goes up. The effectiveness of σ^{70} to bind to core RNAP is impaired due to the activity of the stationary phase-specific protein Rsd, and the small 6S RNA. σ^s has the lowest affinity of all sigma factors for RNAP and its increased synthesis under stress notwithstanding, it never attains more than one-third the level of σ^{70}. Nevertheless, it becomes the most active sigma factor in stressed cells because proteins like Crl, by binding to Eσ^s, greatly enhance its activity. The small nucleotide, guanosine tetraphosphate (ppGpp), has a similar role; this is discussed further below. Certain cell metabolites such as glutamate and acetate may also have a role in stimulating Eσ^s efficiency. The mechanism by which σ^s concentration increases under stress has received a lot of attention and is discussed below.

Ancillary Regulatory Molecules

Cyclic AMP (cAMP)

As stated above, the core stress genes responsible for general resistance are transcribed mainly by Eσ^s and other species of RNAP bound to alternate sigma factors. However, Eσ^{70} does have a role in stress gene expression. The stress genes that this polymerase species transcribes tend to have weak promoters, that is, they deviate from the canonical promoter sequence that Eσ^{70} recognizes. Consequently, the transcription of these genes depends on the availability of ancillary transcriptional factors. This is the case with several starvation genes concerned with uptake of different compounds, and their efficient metabolism when they are present at low concentration. These genes are transcribed if cAMP is available. cAMP binds a protein called CRP, and the resulting complex binds to a specific sequence (AGTGAN$_6$TAACA) present upstream of the promoters of these genes, facilitating transcription by Eσ^{70}. cAMP is present in cells at low concentration under nutrient-sufficient conditions but is increased dramatically during starvation, thereby promoting the transcription of these genes by Eσ^{70}. The cAMP-dependent stress genes, however, play no role in enhanced general resistance, since starved cAMP-deficient strains exhibit the same degree of cross-protection against stresses in general as do cAMP-proficient strains. The role of these genes appears to be confined to the escape response by encoding proteins that enhance the cellular scavenging capacity by improving cellular uptake and metabolic functions.

Given the similarity between the E$\sigma^{70}$and Eσ^s promoters, the following finding is of interest: changing the position of the CRP-binding site in certain genes can alter promoter preference from Eσ^s to Eσ^{70} and vice versa.

Guanosine tetraphosphate (ppGpp)

The small nucleotide ppGpp has been studied intensively in the context of the stringent response, which refers to the phenomenon whereby amino acid starvation results in rapid downregulation of ribosomal RNA (rRNA) biosynthesis and ribosomes. It is now known that the concentration of this nucleotide goes up also in response to starvation for other nutrients as well as in stresses. Its synthesis, initially as pppGpp (which is later dephosphorylated to ppGpp), involves two pathways in *E. coli*: by the ribosome-associated protein RelA, when the ribosome A-site contains an uncharged tRNA during amino acid starvation, and by the protein SpoT, which is responsible for ppGpp synthesis in most other stresses. SpoT can also degrade ppGpp and thus has a dual role. A strain of *E. coli* missing both RelA and SpoT (referred to as ppGpp° strain) cannot synthesize this nucleotide and fails to lower its ribosome production under starvation conditions; such strains are referred to as relaxed strains. In other bacteria, for example, *Streptococcus mutans*, additional enzymes appear to be involved in ppGpp synthesis, such as RelP and RelQ.

In general, ppGpp positively affects the transcription of stress-related genes and negatively those related to growth. It exerts its regulation by binding to $\beta\beta'$ subunits of RNAP near its active site, as has recently been confirmed by crystal structure. This regulation is affected by several mechanisms, such as direct effect on the rate of formation and stability of the open complex, interference with promoter clearance (which obstructs further rounds of transcription), and competition with nucleotide triphosphates used in mRNA synthesis.

A major role of ppGpp in the stress response is that it increases the ability of σ^s (and that of other minor sigma factors) to compete with σ^{70} for binding to the core enzyme. This has been shown in *in vitro* transcriptional assays and is supported by the finding that ppGpp-deficient cells exhibit decreased fractions of both σ^s and σ^{54} bound to the core polymerase. The protein DksA may have a role in augmenting this effect. As can be expected from these findings, absence of ppGpp greatly compromises starvation survival, and proteome and transcriptome analyses have shown that this is because of the lack of stress protein synthesis; instead, the cells continue to express growth-specific proteins. Thus, ppGpp is a necessary adjunct to σ^s for stress survival, and although much of this effect is likely to be affected by ensuring σ^s function, some are likely to be directly due to ppGpp activity.

ppGpp has important roles also in growing cells, where it is required for amino acid synthesis – a deficient strain cannot grow in the absence of exogenously provided amino acids. Further, ppGpp deficiency affects bacterial virulence, for example, expression of genes of pathogenicity islands.

Chemical Alteration in Proteins

Protein phosphorylation

An important mechanism in bacteria for sensing starvation and other stresses, which involves chemical alteration of proteins, is the so-called two-component system. One component of this pair is a histidine protein kinase (HPK) that autophosphorylates at a conserved histidine residue. In response to specific stimuli, the phosphorylated form is stabilized; for this reason, it is also called the 'sensor kinase'. In turn, the HPK phosphorylates the response regulator (RR) protein at a conserved aspartic acid residue. This phosphorylated form of the protein then activates transcription of the target loci. Several pairs of such proteins have been found; these initiate special adaptive strategies in response to specific environmental cues. The HPKs of different systems share homology of about 100 amino acids at their C-terminus; the RRs share homology in the 130 amino acid segments of their N-terminal ends. Among the environmental stimuli sensed by the different two-component systems are phosphate and nitrogen starvations, osmotic changes, and chemotactic stimuli. Here, the phenomenon is illustrated in the context of sensing phosphate starvation.

As stated above (**Table 1**), several genes are induced in response to phosphate starvation; together these genes are referred to as the phosphate regulon. This regulon is under the control of the *phoBR* operon encoding the PhoB and PhoR proteins. The PhoB protein is a positive regulator of this regulon, since

1. Mutations in *phoB*, which inactivate the protein, or deletion of this gene, render the phosphate regulon noninducible.
2. Sequence analysis shows that upstream of the *phoA, phoBR, phoE*, and *pstS* (*phoS*) promoters is a highly conserved 18-bp region (CTNTCATANANCTGTCAN) called the phosphate box. *In vitro* studies demonstrate that purified PhoB protein binds to the phosphate box and that this binding is required for the transcription of the phosphate regulon genes.
3. PhoB bears close homology to the RRs in other systems, such as NtrC (involved in sensing nitrogen starvation) and OmpR (involved in sensing osmotic stress).

The *phoR* gene has a hydropathy profile typical of a membrane protein, and it shows homology to the HPK family of proteins. Like other sensor kinases, it autophosphorylates, a condition that is stabilized by phosphate starvation. It then phosphorylates PhoB, which activates the transcription of the phosphate regulon as discussed above.

Protein oxidation

This type of chemical alteration is involved in activating genes that protect against oxidative stress specifically in response to the ROS, H_2O_2, and O_2^-. A more general mechanism that activates many of the same genes in response to diverse stresses is controlled by σ^s, as discussed above.

H_2O_2 is generally sensed by the transcriptional factor OxyR and O_2^-, by the SoxR/Sox S proteins, although the two systems probably overlap. H_2O_2 directly oxidizes

OxyR. The conserved cysteines, at positions 199 and 208, are in free thiol form in OxyR; H_2O_2 converts them to disulfide form. The resulting conformational change, which has been documented by crystal structure, enables OxyR to activate the transcription of genes involved in escape from oxidative stress (**Table 1**). Upon removal of the H_2O_2 stress, OxyR is reduced by glutaredoxin 1.

The SoxR protein is constitutively synthesized and also becomes activated by direct oxidation, in this case by O_2^-. The protein is a homodimer with two [2Fe-2S] centers per dimer; these centers are the loci of redox changes, that is $[2Fe\text{-}2S]^{1+} \rightleftarrows [2Fe\text{-}2S]^{2+}$ conversion. The oxidized SoxR activates *soxS* gene transcription, which in turn induces a collection of genes called the *soxRS* regulon (**Figure 8**). These genes encode enzymes that can decompose O_2^- (**Table 1**) as well as repair the damage to DNA that may result from oxidative stress, such as the endonuclease IV, mentioned above. At the termination of the stress, SoxR is reduced by an NADPH-dependent SoxR reductase.

Regulation of σ^S Synthesis

As stated above, σ^s is the most important regulatory element in the GSR. Its cellular levels and/or activity increase in response to starvation for diverse individual nutrients as well as other stresses, and how this is accomplished is now understood in some detail at all three levels of control – transcriptional, translational, and posttranslational. I will discuss the results mainly in the context of starvation stress, unless the available information is confined to another stress.

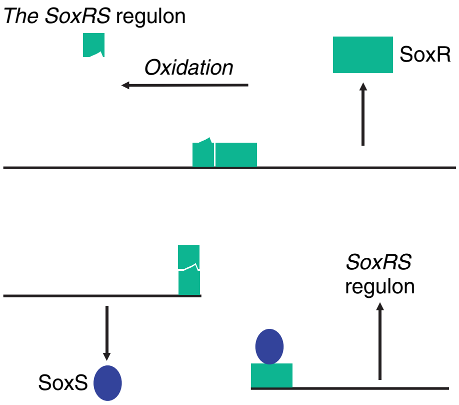

Figure 8 Schematic of SoxRS regulation of the genes involved in defense against O_2^- radical. The change in the configuration of the SoxR protein upon oxidation by O_2^- is schematically represented to show that in its altered configuration, it can activate SoxS transcription, which in turn activates the individual genes of the SoxRS regulon. Reproduced from Matin A (2001) Stress response in bacteria. In: Bolton S (ed.) *Encyclopedia of Environmental Microbiology*, vol. 6, pp. 3034–3046. New York: John Wiley and Sons.

Transcriptional control

The *rpoS* gene is located in an operon downstream of the *nlpD* gene and is transcribed from two promoters, one within the *nlpD* gene and the other upstream of this gene. Use of transcriptional fusions suggested regulation in *E. coli* at this level under starvation, and by ppGpp. However, direct measurement of *rpoS* transcription in *E. coli*, by quantifying the *rpoS* mRNA levels and determination of its half-life, indicates that enhanced transcription has no role in the observed increased levels of this sigma factor in starvation.

Translational control

The main *rpoS* transcript contains an unusually long untranslated region (UTR), which is central to its translational control. The UTR may form two types of hairpin structures. One of these sequesters the translational initiation region (TIR) by pairing with a complementary sequence present within the coding region of the *rpoS* mRNA (called the antisense element), thereby making it unavailable to the ribosomes for translation. Other hairpins may form due to complementary sequences within the UTR. It is possible that both types of secondary structures have a role in regulating *rpoS* mRNA translation, although the involvement of the antisense element-mediated secondary structure in this regulation has not been documented yet. But considerable evidence is available indicating that secondary structures within the UTR minimize *rpoS* translation in unstressed cells and that their relaxation under certain stresses is the major reason for increased cellular σ^s concentration (**Figure 9**). Small noncoding RNA (sRNAs) and the RNA-binding protein, Hfq, play a role in this phenomenon. For example, the sRNA, RprA, possesses a complementary sequence to the UTR stretch of *rpoS* mRNA, which is involved in hairpin formation. Base pairing and hydrogen bonding by this sRNA is able to open the hairpin, free TIR, and permit translation to proceed. Another sRNA, DsRA, is induced under cold stress and promotes *rpoS* translation by a similar mechanism.

Under phosphate starvation, the synthesis of σ^s is regulated at the translational level, but its mechanism is not known. Some five other sRNAs are known to affect *rpoS* translation, but none of these appears to have a role under these starvation conditions. It is possible that an as yet undiscovered sRNA is involved or that the control is exerted through modulation of the antisense element-mediated hairpin. Additional possibilities involve regulation through a variety of proteins that are known to regulate *rpoS* translation. These include the nucleoid protein HU that binds two regions in the *rpoS* mRNA and may influence its secondary structure; the histone-like protein StpA; the cold shock proteins CspC and CspE; a PTS protein; and DnaK.

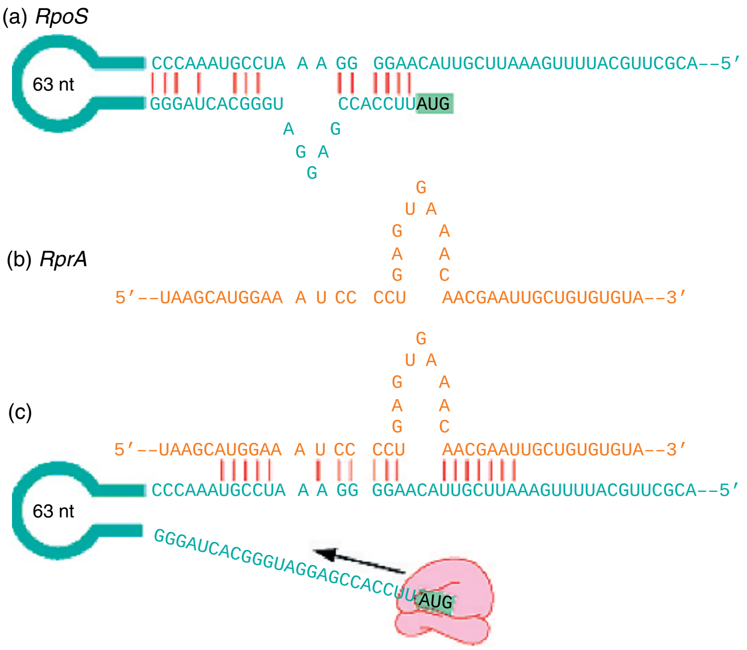
(a) RpoS
63 nt
CCCAAAUGCCUA A A GG GGAACAUUGCUUAAAGUUUUACGUUCGCA--5′
GGGAUCACGGGU CCACCUUAUG
A G
G A
G
(b) RprA
G
U A
G A
A A
G C
5′--UAAGCAUGGAA A U CC CCU AACGAAUUGCUGUGUGUA--3′
(c)
G
U A
G A
A A
G C
5′--UAAGCAUGGAA A U CC CCU AACGAAUUGCUGUGUGUA--3′
63 nt
CCCAAAUGCCUA A A GG GGAACAUUGCUUAAAGUUUUACGUUCGCA--5′
GGGAUCACGGGUAGGAGCCACCUUAUG

Figure 9 The untranslated region (UTR) of the *rpoS* mRNA that encodes σ^s. Note that the sequences upstream of the translational initiation codon (ATG) of the RNA includes regions of internal complementarity that result in the formation of a hairpin structure. This prevents the availability of the initiation codon. The small noncoding RNA, RprA, has regions of homology to the UTR of the *rpoS* mRNA (shown in red; B). Hydrogen bonding between the homologous regions of RprA and *rpoS* mRNA opens the hairpin, permitting translation (C). Reproduced from Matin A and Lynch SV (2005) Investigating the threat of bacteria in space. *ASM News* 71(5): 235–240. Washington, DC: American Society for Microbiology.

Posttranslational control

It was thought that the control of σ^s synthesis in carbon starvation also occurred at the translational level. Direct measurements of *rpoS* mRNA translational efficiency, however, disproved this notion and showed that the increase under these conditions is solely due to enhanced stability of the sigma protein. The experimental results shown in **Table 3** indicate this fact. In this experiment, the rates of *rpoS* mRNA and σ^s synthesis and their half lives were measured, which permitted calculation of the *rpoS* mRNA translational efficiency, that is, the sigma σ^s protein synthesized per unit of the mRNA. *E. coli* cells were cultured in a glucose-limited chemostat in order to precisely establish the relationship between dwindling glucose concentration in the medium (with decreasing dilution rate) and the above mentioned parameters (**Table 3**). As the available glucose diminished, both σ^s synthesis rate and *rpoS* mRNA translational efficiency declined. Meanwhile, however, the stability of the sigma protein increased from 7- to 16-fold, accounting for the observed overall increase in the cellular levels of σ^s.

What accounts for the instability of the sigma protein under carbon-sufficient conditions? The answer came with the discovery that a specific protease, called ClpXP, which is composed of two proteins, ClpX and ClpP, is involved in this regulation. It rapidly degrades σ^s in unstressed cells, but

Table 3 σ^S synthesis rate and *rpoS* mRNA translational efficiency in glucose-sufficient cells and those subjected to increasing degree of glucose starvation (last three rows)

Glucose concentration (M)	σ^S *Concentration*[a]	σ^S *half-life (min)*	σ^S *synthesis rate*[b]	rpoS *mRNA concentration*[c]	rpoS *translational efficiency*[d]
10^3 (glucose sufficiency)	190	5	55	1.0	1.0
2.2×10^6	270	11	34	0.75	0.75
1.3×10^6	300	34	13	0.52	0.52
1.2×10^6	570	>60	ND	0.5	0.5

[a] pmol mg^{-1} cell protein. [b] pmol per mg cell protein per min. [c] Relative units. [d] σ^S synthesis rate/*rpoS* mRNA concentration.
ND, not determined.
Reproduced from Zgurskaya HI, Keyhan M, and Matin A (1997). The σ^S level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Molecular Microbiology* 24(3): 643–651.

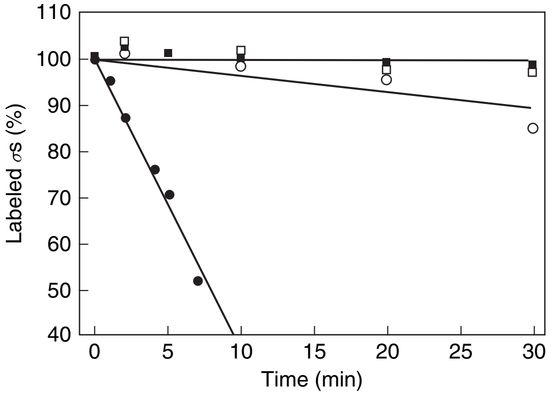

Figure 10 Comparison of σ^s stability in exponential phase (solid symbols) and stationary phase (open symbols) cultures in *clpP*-proficient (circles) and *clpP*-deficient (squares) backgrounds. Note that in a wild-type background, σ^s is stable only in the stationary phase, but in a mutant missing the Clp protein, it is stable in both the phases of growth. Reproduced from Schweder T, Kyu-ho L, Lomovskaya O, *et al*. (1996) Regulation of *Escherichia coli* starvation sigma factor (σ^2) by ClpPX protease. *Journal of Bacteriology* 178(2): 470–476.

not in those experiencing carbon starvation (**Figure 10**). ClpP is a double-ring peptidase with 14 active sties on the inside of the ring. The hexameric rings of ClpX bind to one or both ends of the ClpP chamber. The target proteins are recognized by ClpX, which unfolds the proteins to be degraded using ATP and feeds them into the ClpP chamber (**Figure 11**). Remarkably, despite the fact that the bacterial cell also contains several other proteases, ClpAP, Lon, HslUV, and FtsH, σ^s is degraded only by the ClpXP protease. The stretch between 173 and 188 amino acids within the σ^s protein is required for its recognition as a ClpXP target. ClpX targets proteins containing an 11-amino acid stretch at their N- or C-terminal ends, called the ssrA tag, and may unfold the target proteins by acting on this tag. The stability of the protein structure adjacent to the tag also appears to have a role – the less stable this structure, the easier it is for ClpXP to degrade a protein.

If ClpXP protease can degrade σ^s in exponential phase cells, why does this protein become resistant to this protease in the stationary phase? Another protein, SprE (RssB), has a role in this phenomenon. SprE is a homologue of RR proteins, mentioned above, but is unique in its C-terminal output domain and in the fact that it controls the stability of a protein, namely σ^s. SprE forms a quaternary complex with ClpP, ClpX, and σ^s, and this complex can degrade the sigma protein *in vitro*. SprE is active in exponential phase cells, but becomes inactive under carbon starvation, and this is thought to account for the fact that σ^s stability increases under these conditions. By analogy to other RRs, it was assumed that SprE is active in its phosphorylated state, but the search for a cognate sensor kinase (see above) has remained elusive. According to some researchers, SprE may be phosphorylated by several different kinases or small molecule phosphate donors. According to others, however, phosphorylation at the conserved aspartate of SprE may not be necessary for its activity. It was shown that SprE, in which the conserved aspartate is mutated, still retains full activity. What activates SprE remains unknown.

Activity control

Control at the level of activity of σ^s evidently operates in nitrogen starvation. Under these conditions, the core set of proteins are still synthesized even though σ^s levels show only a very modest increase. Thus, it is thought

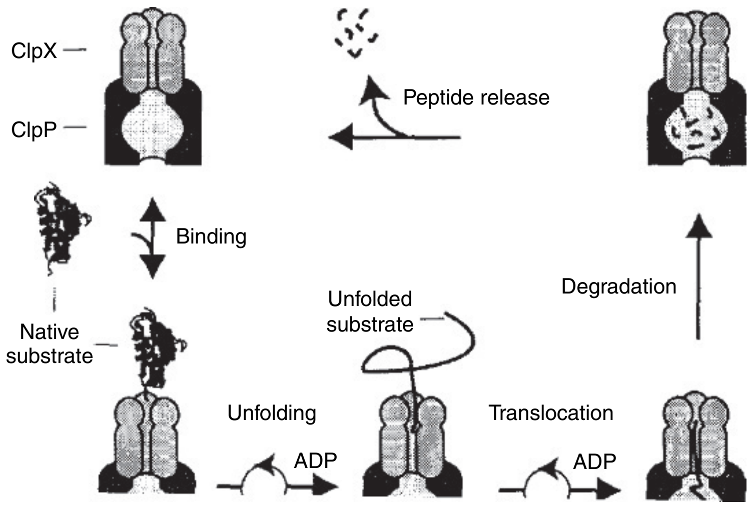

Figure 11 Schematic representation of native protein degradation by ClpXP protease. The ClpX component of the protease binds the substrate protein and unfolds it by its ATPase activity. The unfolded protein is translocated through the ClpP chamber, a process that also requires ATP, and is degraded; the resulting peptide fragments are released. Reproduced from Kenniston JA, Burton RE, Siddique SM, *et al*. (2004) Effect of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation. *Journal of Structural Biology* 146: 130–140.

that the sigma protein is more active under these conditions. The factors that may account for this are hypothesized to be those that increase the competitiveness of σ^s for RNAP. These have been discussed above (see 'Other factors involved in favoring Eσ^s-mediated transcription').

Regulation under low-shear/simulated microgravity conditions

As alluded to above, low-shear environments, such as brush border microvilli of the gastrointestinal, respiratory, and urogenital tracts, are common routes of microbial infection. Low shear environments closely resemble microgravity conditions experienced by astronauts during space flight. There has therefore been considerable interest in studying the biological effects of these conditions. On Earth, the effects of such environments are simulated by the use of a special cultivation equipment that utilizes high aspect to ratio vessels (HARVs). Early studies strongly indicate that these conditions weaken the human immune response and make bacteria more virulent and stress-tolerant; these have obvious implications for the control of disease on Earth and astronauts' health. Studies on the regulation of this phenomenon have resulted in some intriguing findings. Thus, the increased bacterial resistance that low-shear environments confer on bacteria appears to be independent of σ^s in exponential but not in stationary phase. Further, these environments markedly enhance *rpoS* translational efficiency regardless of the growth phase and promote σ^s instability, especially in the exponential phase. Since both these regulatory phenomena involve macromolecular folding pattern, the findings raise the possibility that low-shear/microgravity environments can influence these patterns. That microgravity conditions make bacteria more virulent has recently been confirmed in experiments involving bacterial growth in space.

Sensing starvation

Given that the regulation of the starvation response differs depending on the missing nutrient, it seems likely that the dearth of different nutrients is sensed by different mechanisms. The sensing mechanism in the case of carbon starvation could be an effector that inactivates SprE or ClpXP. Recent reports indicate that an increase in denatured proteins may have a role. Starvation affects fidelity of ribosomes, resulting in the synthesis of abnormal proteins with a proclivity for oxidation. The latter sequester Clp, impairing ClpXP activity, resulting in the stabilization of σ^s. In this view, starvation is sensed by the increase in aberrant proteins. Phosphate and nitrogen starvations may involve the PhoBR- and NtrBC-sensing systems mentioned above. In *P. putida*, a G-protein, called FlhF, which is situated at the cell pole, may be involved in sensing stress, as its absence robs the cell of the capacity to develop the general stress resistance.

Concluding Remarks

It is evident that in response to hostile and frequently fluctuating conditions in nature, bacteria have evolved highly sophisticated mechanisms that permit them to swiftly shift between rapid growth and static survival modes. Our understanding of this phenomenon has enhanced greatly in the last two decades, and further progress is likely to yield information that will permit better control of bacterial growth – its enhancement toward beneficial ends, such as ecosystem management, industrial processes, and bioremediation, as well as its mitigation as in disease.

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Transcriptional Regulation

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Defining Statement
The Transcription Machinery
Template Recognition: Promoters
Transcription Initiation
Transcription Elongation
Transcription Termination
Further Reading

Glossary

−10 element A consensus sequence centered about 10 bp before the start point of transcription that is involved in the initial melting of DNA by RNA polymerase.

−35 element A consensus sequence centered about 35 bp before the start point of transcription that is involved in the initial recognition by RNA polymerase.

promoter A sequence of DNA whose function is to be recognized by RNA polymerase to initiate transcription. A typical *Escherichia coli* promoter contains two conserved elements, a −10 element and a −35 element (see above).

RNA polymerase The enzyme that synthesizes RNA using a DNA template (also termed DNA-dependent RNA polymerase).

sigma (σ) factor A subunit of bacterial RNA polymerase needed for initiation. The σ factor has major influence on the selection of promoters.

start point The position on the DNA that corresponds to the first base transcribed into RNA.

terminator A DNA sequence that causes RNA polymerase to terminate transcription and to dissociate from the DNA template.

transcription The synthesis of RNA on a DNA template.

transcription factor A protein needed to activate or repress transcription but is not part of the RNA polymerase enzyme.

transcription unit The DNA sequence that extends from the promoter to the terminator; it may include more than one gene.

Abbreviations

CAP	catabolite gene-activator protein
IHF	integration host factor
mRNA	messenger RNA
Nus	N utilization substance
RNAP	RNA polymerase
rRNA	ribosomal RNA
tRNA	transfer RNA

Defining Statement

The first step in gene expression is the transcription of the coding DNA sequences to discrete RNA molecules. Specific DNA regions, defined as promoters, are recognized by the transcribing enzyme, a DNA-dependent RNA polymerase (RNAP). The RNAP binds to the promoter and initiates the synthesis of the RNA transcript. The enzyme catalyzes the sequential addition of ribonucleotides to the growing RNA chain in a template-dependent manner until it comes to a termination signal ('terminator'). The DNA sequence between the start point and the termination point defines a 'transcription unit'. An RNA transcript can include one gene or more. Its sequence is identical to one strand of the DNA, the coding strand, and complementary to the other, which provides the template. The base at the start point is defined as +1 and the one before that as −1. Positive numbers are increased going downstream (into the transcribed region), whereas negative numbers increase going upstream. The immediate product of transcription, which extends from the promoter to the terminator, is termed as 'primary transcript'. In prokaryotes, messenger RNA (mRNA) is usually translated concomitantly while being transcribed and is rapidly degraded when not protected by the ribosomes, whereas ribosomal RNA (rRNA) and transfer RNA (tRNA) are cleaved to give mature products and are stable.

Transcription is the principal step at which gene expression is controlled. Many factors, such as DNA signals, regulatory proteins, noncoding RNAs, and small ligands (nucleotides, metabolites), determine whether the polymerase will choose to transcribe a certain gene and whether the whole process of transcription will be accomplished successfully. The timing of transcription of specific genes is influenced by environmental conditions and by the growth cycle phase.

The molecular picture of how genes are transcribed and the nature of the regulatory mechanisms that control transcription are far from being complete, but lots of progress has been made. Work on relatively simple organisms, bacteriophage, and bacteria has provided new insights into the mechanisms that are involved in the regulation of gene expression, transcriptional control mechanisms being among them. Although there are significant differences in the organization of individual genes and in the details and the complexity of the regulatory mechanisms among prokaryotes and eukaryotes, it is clear that basic principles are shared among all organisms. Due to the relative simplicity of prokaryotic biochemical pathways, their easy manipulation in the laboratory, and the advanced tools that are available for changing their genotype and for testing the resulting phenotype, it is easier to infer these basic principles by studying prokaryotes.

The Transcription Machinery

RNA Polymerase Catalyzes Transcription

RNA synthesis is catalyzed by the enzyme RNA polymerase (RNAP). The reaction involves the incorporation of ribonucleoside 5′ triphosphate precursors into an oligoribonucleotide transcript, based on their complementarity to bases on a DNA template. RNAP catalyzes the formation of phosphodiester bonds between the ribonucleotides. The formation of a phosphodiester bond involves a hydrophilic attack by the 3′-OH group of the last ribonucleotide in the chain on the 5′ triphosphate of the incoming ribonucleotide. The incoming ribonucleotide loses its terminal two phosphate groups, which are released in the form of pyrophosphate. In this manner, the RNA chain is synthesized from the 5′ end toward the 3′ end.

Multisubunit RNAPs are remarkably conserved in fundamental structure and mechanism. The best characterized RNAPs are those of eubacteria, for which *Escherichia coli* is a prototype. Unlike eukaryotic cells, in which various types of polymerases are dedicated to the synthesis of the various types of RNA, in eubacteria a single type of polymerase appears to be responsible for the synthesis of messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA).

The dimensions of bacterial RNAP are approximately 90–95–160 Å. The molecular weight of the complete *E. coli* enzyme is approximately 465 kDa. About 7000 molecules of RNAP are present in an *E. coli* cell, but the number of molecules engaged in transcription at any given time varies from 2000 to 5000, depending on the growth conditions.

The DNA sequence that is being transcribed by RNAP is transiently separated into its single strands, with one of the strands serving as a template for the synthesis of the RNA strand. This region is therefore defined as the 'transcription bubble'. As the RNAP moves along the DNA, it unwinds the duplex at the front of the bubble and rewinds the DNA at the back, so that the duplex behind the transcription bubble reforms. Thus, the bubble moves with the RNAP, and the RNA chain is elongated. The length of the transcription bubble varies from 12 to 20 bp. The length of the transient hybrid between the DNA and the newly synthesized RNA sequence within the transcription bubble is a matter of controversy and the estimates range from 2 to 12 nt. Beyond the growing point, the newly synthesized RNA chain enters a high-affinity binding site within the RNAP.

Bacterial RNA Polymerase Consists of Multiple Subunits

The RNAPs of certain phage consist of single polypeptide chains. These polymerases recognize a very limited number of promoters and they lack the ability to change the set of promoters from which they initiate transcription. In contrast, bacterial RNAPs consist of several subunits. The most studied bacterial RNAP, the one in *E. coli*, exists in two forms, an holoenzyme and a core enzyme. The holoenzyme is capable of selective initiation at promoter regions, whereas the core RNAP is capable of elongation and termination, but not selective initiation. The two forms of the polymerase consist of two identical α-subunits, one β-subunit, and one β'-subunit, but only the holoenzyme contains an additional subunit, one of the several σ proteins. The subunit composition of the holoenzyme is summarized in **Figure 1**. The α-subunit plays an important role in RNAP assembly, which proceeds in the pathway $\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta\beta' \rightarrow \alpha_2\beta\beta'\sigma$. The α-subunit plays some role in promoter recognition and in the interaction of RNAP with transcriptional activators (see later). The β and β' subunits together constitute the catalytic center of RNAP. The β-subunit was demonstrated to contact the template DNA, the newly synthesized RNA, and the substrate ribonucleotides. The β'-subunit contacts the RNA chain as well. Mutations in the genes that encode β and β' show that both subunits are involved in all stages of transcription. The sequences of β and β' show homology to the sequences of the largest subunits of eukaryotic RNAPs. This conservation through the evolution hints that the mechanisms by which all RNAPs catalyze transcription share common features. In accord, high-resolution structural studies on the core enzyme show that it adopts a certain structure, which is similar to the structure that is found in the yeast RNAP II. The assignment of individual functions to the different subunits of the core polymerase is only a rough

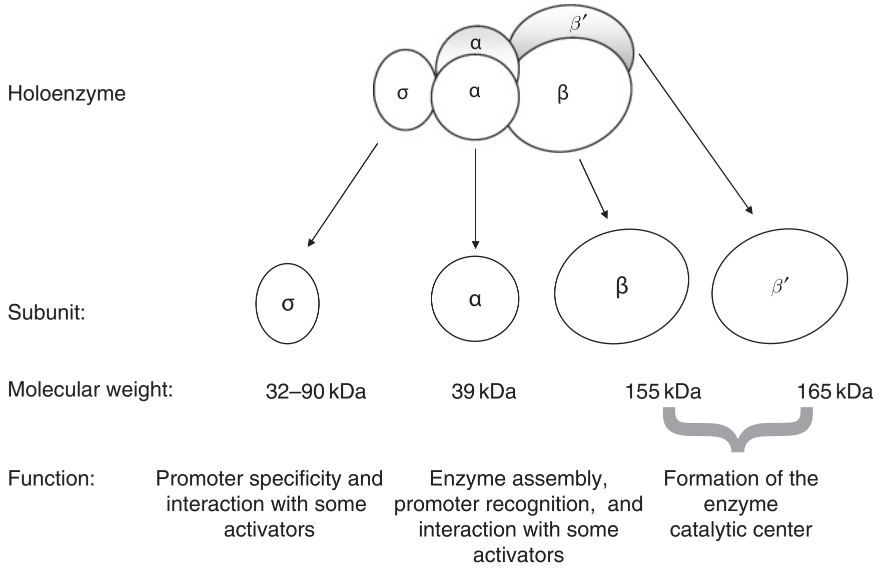
Holoenzyme
σ
α
α
β
β′
Subunit:
σ
α
β
β′
Molecular weight:
32–90 kDa
39 kDa
155 kDa
165 kDa
Function:
Promoter specificity and interaction with some activators
Enzyme assembly, promoter recognition, and interaction with some activators
Formation of the enzyme catalytic center

Figure 1 *E. coli* RNA polymerase holoenzyme consists of four types of subunits.

estimation because most probably each subunit contributes to the activity of the enzyme as a whole. In addition, a small 91-residue protein, termed the ω subunit, associates with the polymerase. It has no direct role in transcription, but seems to function as a chaperone to assist the folding of the β' subunit.

The σ subunit has three main functions: to ensure the recognition of specific promoter sequences; to position the RNAP holoenzyme at a target promoter; and to facilitate unwinding of the DNA duplex near the transcript start site. Based on recent studies, 'σ' subunits are involved also in other aspects of transcription initiation, such as abortive initiation and promoter escape. There are several types of σ factors in the bacterial cell (see later). The major σ factor in *E. coli*, which is required for most transcription reactions, is σ^{70}. Although σ^{70} has domains that recognize the promoter DNA sequence, as an independent protein, it does not bind to DNA, perhaps because its DNA-binding domain is sequestered by another domain of the σ^{70} molecule (when σ^{70} is shortened from its N-terminus, it is able to bind to DNA, suggesting that the N-terminal region has an inhibitory effect on the ability of σ^{70} to bind to DNA). However, upon binding to the core enzyme and the formation of the holoenzyme complex, σ^{70} undergoes a conformational change and it now contacts the region upstream of the start point. The dogma is that the σ factor discharges from the core enzyme when abortive initiation is concluded and RNA synthesis is successfully initiated (although findings suggest that, at least in some cases, σ can remain associated with the polymerase during postinitiation steps). The released σ factor becomes immediately available for use by another core enzyme, although the activity of many σ factors is controlled by an anti-σ factor, which sequesters it away from the RNAP. *E. coli* cells contain about 3000 molecules of σ^{70}, enough to bind about one-third of the intracellular core RNAP.

The Ability of RNA Polymerase to Selectively Initiate Transcription is Dependent on the Presence of σ Factor

Bacterial polymerases have to recognize a wide range of promoters and to transcribe different genes on different occasions. Specificity of gene expression is in part modulated by substituting one species of σ for another, each specific for a different class of promoters. In *Bacillus subtilis*, σ factors are implicated in the temporal regulation of sporulation. In *E. coli*, alternative σ factors are used to respond to general environmental changes. The σ factors are named either by their molecular weight (e.g., σ^{70}) or after their genes, which are usually termed 'rpo' (e.g., RpoD for σ^{70}).

When cells are shifted from low to high temperature, the synthesis of a small number of proteins, the heat-shock proteins, transiently increases. The σ^{32} protein (RpoH) is responsible for the transcription of the heat-shock genes. The basic signal that induces the production of σ^{32} is the accumulation of unfolded proteins that results from the increase in temperature. The heat-shock proteins play a role in protecting the cell against environmental stress. Several of them act like chaperones, preventing the unfolding (denaturation) of proteins. The heat-shock proteins are synthesized in response to other conditions of stress, implying that the production of σ^{32} is induced also by conditions other than elevation in temperature. The σ^{32} protein is unstable and it is rapidly degraded when it is not needed. Another σ factor, σ^{E}, appears to respond to more extreme temperature shifts that lead to the accumulation of unfolded proteins, which are usually found in the periplasmic space or the outer membrane. Less is known about this σ factor and about the genes it controls. When *E. coli* and other enteric bacteria are nitrogen-limited, the synthesis of a number of proteins is dramatically induced. The increased production of these proteins enlarges the capacity of cells to produce nitrogen-containing compounds, and to use nitrogen sources other than ammonia. The transcription of the genes that encode these proteins is dependent on σ^{54} (also known as σ^{N}). Expression of the flagellar genes under normal conditions depends on σ^{28} (also known as σ^{F}).

When *E. coli* cells are starved, they shift from the exponential-growth phase to the stationary phase. The ability of the cells to cope with starvation depends on the production of many proteins. This is enabled due to the synthesis of σ^{S} (RpoS), which transcribes the relevant genes. Unlike *E. coli*, when *B. subtilis* cells are starved, they can form spores. Sporulation involves the differentiation of a vegetative bacterium into a mother cell and a forespore; the mother cell lyses and a spore is released. The process of sporulation involves a drastic change in the biosynthetic activities of the bacterium, in which many genes are involved. This complex process is concerted at the level of transcription. The principle is that in each compartment (the mother cell and the forespore) the existing σ factor is successively displaced by a new σ factor that causes the transcription of a new set of genes. Communication between the compartments occurs in order to coordinate the timing of the changes in the mother cell and the forespore.

The promoters identified by the various σ factors are organized similarly, having their important elements centered around 10 and 35 nt upstream from the start point (see later), except for σ^{54}, whose promoters have slightly different characteristics. However, the ability of different σ factors to cause RNAP to initiate at different sets of promoters stems from the fact that each type of σ factor recognizes promoter elements with unique sequences. σ^{54} differs from the other σ factors also in its ability to bind to DNA independently, and by the influence of sites that are distant from the promoter on its activity. In many aspects, σ^{54} resembles eukaryotic regulators.

A comparison of the sequences of the different σ factors identifies four regions that have been conserved. Several sequences in these regions were identified with individual functions, such as interaction with core RNAP or contacting the various promoter elements. Recent structural information shows that two key σ domains are structurally conserved, even among diverse family members.

Template Recognition: Promoters

Promoter Recognition Depends on Conserved Elements

Template recognition begins with the binding of RNAP to the promoter. Promoter is a sequence of DNA whose function is to be recognized by RNAP to initiate transcription. The information for promoter function is provided directly by the DNA sequence, unlike expressed regions, which require that the information be transferred into RNA or protein to exercise their function. Two main approaches were used to identify the DNA features that characterize a promoter. The first is comparative sequence analysis and the second is the identification of mutations that alter the recognition of promoters by RNAP. Comparison of the sequence of many *E. coli* promoters recognized by the major RNAP species, σ^{70} holoenzyme, revealed an overall lack of extensive conservation of sequence over the 60 bp associated with RNAP. Nevertheless, statistical analysis revealed some commonalities. A typical *E. coli* promoter that is recognized by σ^{70} contains four conserved features: the start point, the −10 region, the −35 region, and the distance between the −10 and −35 regions. The start point is usually a purine. It is often the central base in the sequence CAT, but this is not a mandatory rule. The −10 region is a hexanucleotide that centers approximately 10 bp before the start point, although this distance is somewhat variable. Its consensus sequence is TATAAT (in the antisense strand). The conservation is T80 A95 T45 A60 A50 T96, where the numbers refer to the percent occurrence of the most frequently found base at each position. The −35 region is a hexanucleotide sequence that centers approximately 35 bp upstream of the start point. Its consensus is TTGACA and the conservation is T82 T84 G78 A65 C54 A45. The favored spacing between the −10 and the −35 sequences is 17 bp.

For most promoters, there is a good correlation between promoter strength and the degree to which the −10 and −35 elements agree with the consensus sequences. The significance of the conserved promoter features was further emphasized by the finding that most mutations that alter promoter activity (i.e., affect the level of expression of the gene(s) under the control of this promoter) change the sequence of the particular promoter in an expected fashion. Mutations that increase the similarity to the proposed conserved −10 and −35 sequences or bring the spacing between them closer to 17 bp, usually, enhance the promoter activity (‘up mutations’); mutations that decrease the similarity to the conserved sequences or bring the spacing between them more distant than 17 bp, usually, reduce the promoter activity (‘down mutations’). The nature of down mutations in the −35 and −10 regions of various promoters led to the conclusion that the −35 region is implicated in the recognition of the promoter by RNAP and the formation of a closed transcription complex, whereas the −10 region is implicated in the shift of the closed complex to the open form (see later). The fact that the −10 region is composed of AT base pairs that require low energy for melting makes it suitable to assist in unwinding and, thus, in converting the transcription complex into its open form.

There are several exceptions to the proposed generalized pattern. For example, some promoters lack one of the conserved sequences, the −10 or the −35 region, without a corresponding effect on promoter activity. In some cases, it was proposed that another sequence compensates for the lack of a consensus sequence. In still other cases, it was concluded that the promoter cannot be recognized by RNAP alone, and the involvement of additional proteins that overcome the deficiency in intrinsic interaction between RNAP and the promoter is required. Other exceptional promoters were discovered due to the isolation of promoter mutations that do not affect any of the conserved promoter features described so far. Rather, they are the outcome of base substitutions in other sequences in the vicinity of the conserved sequences or the start point. One explanation for these findings is that the analysis that generated the sequence characteristics of a typical promoter may have missed some sites that contribute to the transcription initiation process, possibly because it included too many promoters, both weak and strong. Alternatively, other base pairs in the promoter could be recognized by RNAP, but might become significant only if canonical recognition sites are absent.

The isolation of deletions that progressively approach specific promoters from the upstream region demonstrated the involvement of specific upstream sites in the recognition of RNAP in some cases. This led to the discovery that some *E. coli* promoters contain a third important element in addition to the −10 and −35 sequences. This element was named the upstream element, or 'UP element', because it is located approximately 20 bp upstream of the −35 region. Its sequence is AT-rich and it was first identified in the strong promoters of the *rrn* genes, which encode rRNA. It is believed now that promoter strength is a function of all three elements, −10, −35, and UP, with very strong promoters, such as the *rrn* promoters, having all three elements with near-consensus sequences and with weaker promoters having one, two, or three nonconsensus promoter elements. It has been found that, whereas −70 is responsible for the recognition of the −10 and −35 regions, the UP element interacts with the α-subunit of RNAP.

Finally, the activity of some promoters is affected by sequences downstream to the −10 region, or even downstream to the transcription start point. The sequences immediately around the start point seem to influence the initiation event. The effect of the initial transcribed region (from +1 to +30) on promoter strength is explained by the influence of this region on the rate at which RNAP clears the promoter. Unlike the promoters described so far, which are recognized by holoenzymes that contain σ^{70} or a close homologue, a minority of the cellular holoenzymes use σ^{54} and have different basal elements located at −12 and −24. Transcription initiation from these promoters relies also on enhancer-like elements that are remote (upstream) from the promoters (see later).

Possible Mechanisms of Promoter Recognition

How does RNAP find the promoter sequences? How does it identify a stretch of ~60 bp that defines a promoter in the context of 4×10^6 bp that make up the *E. coli* genome? Three models were suggested to explain the ability of RNAP to find promoters. The first model assumes that RNAP moves in the cell by random diffusion. It associates and dissociates from loose binding sites on the DNA until, by chance, it encounters a promoter sequence that allows tight binding to occur. According to this model, movement of an RNAP molecule from one site on the DNA to another is limited by the speed of diffusion through the medium. However, this parameter might be too low to account for the rate at which RNAP finds promoters. The second model addresses this problem by assuming that once an RNAP molecule binds to a DNA sequence, the bound sequence is directly displaced by another sequence; the enzyme exchanges sequences very rapidly, until it binds to a promoter that allows an open complex to form and transcription initiation to occur. According to this model, the association of the polymerase with DNA sequences and their dissociation are essentially simultaneous. Thus, the time spent on site exchange is minimal and the search process is much faster in comparison to the speed calculated based on the first model. This model fits the accepted notion that core polymerases that are not busy in transcription are stored by binding to loose sites on the DNA. The third model assumes that RNAP binds to a random site on the DNA and starts sliding along the DNA molecule until it encounters a promoter. The actual mechanism by which RNAP finds promoters might combine features of the various models.

Transcription Initiation

The activities of genes are frequently regulated at the initiation step of transcription. Therefore, the initiation of transcription is a very precise event that is tightly controlled by various regulatory mechanisms. Studying transcription initiation in *E. coli* has served as a model for understanding transcriptional control throughout all kingdoms of life.

Stages of the Transcription Initiation Process

Transcription initiation is the phase during which the first nucleotides in the RNA chain are synthesized. It is a multistep process that starts when the RNAP holoenzyme binds to the DNA template and ends when the core polymerase escapes from the promoter after the synthesis of approximately the first nine nucleotides.

The stages of the transcription initiation process, which are summarized in **Figure 2**, can be described in terms of the types of interaction between the RNAP and the nucleic acids that are involved. The first stage in transcription initiation is the formation of a complex between the holoenzyme and the DNA sequence at the promoter, which is in the form of a double-stranded DNA. This complex is termed a closed binary complex or *closed complex*. The second stage is the unwinding of a short region of DNA within the sequence that is bound to the RNAP. The complex between the polymerase and the partially melted DNA is termed an open binary complex or *open complex*. The conversion of the closed complex into the open complex leads to the establishment of tight binding between the RNAP and the promoter sequence. For strong promoters, the conversion into an open complex is irreversible. The third stage is the incorporation of two ribonucleotides and the formation of a phosphodiester bond between them. Because the complex at that stage contains an RNA as well as DNA, it is called an initiation *ternary complex*. Up to seven additional ribonucleotides can be added to the RNA chain without any movement of the polymerase. After the addition of each base, there is a certain probability that the enzyme will release the short (up to nine bases long) RNA chain. Such an unsuccessful initiation event is termed an 'abortive initiation'. Following an abortive initiation, RNAP begins again to incorporate the first base. Several rounds of abortive initiations usually occur and the result is the formation of short RNA chains that are 2–7 bases long. When initiation succeeds, that is, a nine-base-long RNA chain is formed and is not released, the last stage in transcription initiation occurs. At that stage the σ factor is released from the polymerase. As a consequence, a complex containing core polymerase, DNA, and RNA is formed. This complex is called an elongation ternary complex. The departure of the polymerase from the promoter to resume elongation is termed promoter escape or *promoter clearance*.

Transcription Factors

Regulation of transcription involves a complex network, where DNA-binding proteins, termed transcription factors, are a key component. Transcription factor is a protein needed to activate or repress the transcription of a gene, but is not itself a part of the enzyme. Some transcription factors bind to *cis*-acting DNA sequences only; some bind to each other; others bind to DNA as

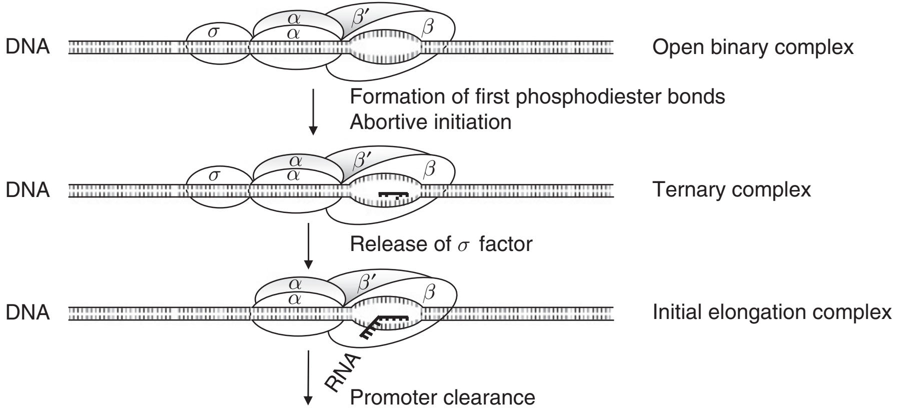

Figure 2 Stages of the transcription initiation process.

well as to other transcription factors. When a transcription factor binds to a specific promoter, it can either activate or repress transcription initiation. Some transcription factors function solely as activators or repressors, whereas others can function as either according to the target promoter.

The *E. coli* genome contains more than 300 genes that encode proteins that are predicted to bind to promoters, and to either up- or down-regulate transcription. So far, about half of these have had their functions verified experimentally. Some of these proteins control large numbers of genes, whereas others control just one or two genes. It has been estimated that seven transcription factors (CRP, FNR, IHF, Fis, ArcA, NarL, and Lrp) control 50% of all regulated genes, whereas ~60 transcription factors control only a single promoter. Bacterial transcription factors can be grouped into different families on the basis of sequence analysis. So far, a dozen families have been identified, the best characterized of these being the LacI, AraC, LysR, CRP, and OmpR families. Because a large percentage of the polymerase molecules are occupied with transcription of ribosomal genes, transcriptional regulation of these genes is intensively studied. It has been shown that, in addition to σ^{70}-RNAP, the Fis and H-NS proteins bind near the upstream P1 promoter of the seven ribosomal transcription units and regulate transcription.

The activity of most bacterial promoters is dependent on multiple environmental cues, rather than just one signal. In most cases, for a promoter to respond to multiple signals, multiple transcription factors are required. Accordingly, many promoters are controlled by two or more transcription factors, with each factor relaying one environmental signal. However, in some cases a regulatory protein can 'integrate' multiple signals, like the NifL–NifA system in *Azotobacter vinelandii*, which controls the genes that are involved in nitrogen fixation in response to oxygen levels and the availability of carbon and nitrogen. There are a few examples of integrated regulation that are solely dependent on repressors, but, in most examples studied so far, complex regulation depends on combinations of repressors and activators or codependence on more than one activator.

Repression of Transcription Initiation

Gene expression is sometimes negatively regulated by a repressor protein that, when bound to DNA, inhibits transcription initiation. The ability of the repressor to bind to DNA is in turn modulated by the binding of an effector molecule to the repressor. The regulation of the *lac* operon expression in *E. coli* is a paradigm for this type of transcriptional control. LacI is a repressor protein that blocks the initiation of transcription from the promoter of the *lac* operon. LacI binds to a site on the DNA, termed an *operator*, that overlaps with the promoter. Because of this overlap, the binding of the LacI repressor and of RNAP are competitive events. That is, RNAP cannot bind to the promoter until the repressor is removed from the operator. The binding of one of several β-galactoside compounds to the repressor destabilizes the repressor–operator complex and allows RNAP to bind to the promoter to initiate transcription. Interestingly, the *lac* operon has two additional binding sites for LacI, an upstream site and a downstream site, located in the first gene of the operon. Compared to the operator, the additional sites have a lower affinity for the repressor protein and it was suggested that they do not directly participate in the inhibition of transcription initiation. Rather, the secondary binding sites seem to stabilize the repressor–operator complex.

There are important exceptions to the *lac* repression paradigm. An example is the repression of the *gal* operon transcription initiation by a different and less understood mechanism. The *gal* operon contains two repressor-binding sites, both required for maximum efficacy of the *gal* repressor, yet neither of these operators overlaps with the promoter sequences. Thus, the binding of the *gal* repressor to its operators does not seem to compete directly with binding of RNAP to the *gal* promoter. It was suggested that the *gal* repressor, when bound to both binding sites, holds the DNA in a conformation that is unfavorable for binding to RNAP.

Activation of Transcription Initiation

The frequency of transcription initiation from many promoters is enhanced by activator proteins. Activators improve the performance of a promoter by improving its affinity for RNAP. In most cases, they bind within or upstream from the promoter and make a direct contact with RNAP. The activators that interact with the most abundant form of RNAP involved in transcription initiation in *E. coli*, the σ^{70} holoenzyme, can be roughly divided into two groups, those that interact with the α-subunit of RNAP and those that interact with the σ^{70} subunit.

The best characterized activator from the first group is the catabolite gene-activator protein (CAP). The CAP target site on the DNA was determined in a number of systems. Comparative sequence analyses led to the definition of a consensus sequence for CAP binding. CAP-binding sites are found at various locations relative to the transcription start point in different systems. The most studied case is the activation of transcription initiation from the *lac* promoter by CAP. The regions that are required for the activation on both CAP and the α-subunit of RNAP were defined. CAP acts as a dimer, and although the activating region is present in both subunits of the CAP dimer, transcription activation at the *lac* promoter requires only the activating region of the promoter-proximal subunit. CAP interacts with the C-terminal domain of the α-subunit (α CTD). The α CTD constitutes an independently folded domain, which is connected to the remainder of α by a flexible linker. This allows α CTD to make different interactions in different promoters. The simplest model for transcription activation by CAP is that CAP binds to the DNA and recruits the α CTD, and thus the RNAP holoenzyme, to the promoter.

The best characterized activator from the second group is the cI protein of bacteriophage λ (λcI). λcI binds to a site on the DNA that overlaps the −35 element of the λP_{RM} promoter. The activating region in λcI was defined and was demonstrated to directly contact a specific region in σ^{70}.

The existence of at least two groups of activators that bind to separate targets on the DNA and to different components of RNAP raised the possibility, which was later proven, that, at some promoters RNAP might be contacted simultaneously by two or more activators. Generally, where two transcription factors are involved, one factor interprets a global metabolic signal, whereas the other responds to a specific metabolic signal. The best illustration of this is the *E. coli lac* promoter, which is regulated by CRP that depends on a global signal, glucose starvation, and by the Lac repressor, which is controlled by a specific metabolite, allolactose. In most cases, multiple activators bind independently to their target promoters. However, there are a few promoters at which activators bind cooperatively.

In contrast to the copious σ^{70} promoters, the rare σ^{54} promoters, which contain −12 and −24 basal elements instead of the well-known −10 and −35 elements, seem to be regulated solely by activation rather than by repression. σ^{54} activators (the most studied is NtrC) bind to enhancer-like sites on the DNA; that is, the sites are remote from the promoters (upstream) and their precise location is not critical for transcriptional activation. In fact, these sites can be moved kilobases in *cis* and retain their residual function. Thus, unlike σ^{70} activators that bind to sites that enable direct communication with RNAP, σ^{54} activators, once bound to their DNA target sites, cannot touch the polymerase without looping out the intervening DNA. This seems to be the reason why σ^{54} promoters frequently require the help of integration host factor (IHF), which enhances the bending of the DNA, as a cofactor. It is accepted that σ^{54} polymerase can bind to its promoters to form a closed complex. However, this polymerase cannot transcribe because it cannot melt the DNA. Once the upstream activator binds to its target site upstream of the promoter, it loops out of the sequence between its binding site and the promoter and touches the complex. This interaction triggers the melting of DNA (with the help of a helicase activity) and the creation of a transcription bubble. Thus, σ^{54} activators catalyze the conversion of the polymerase–promoter complex from a closed state to a transcription-ready open state, rather then tethering the RNAP to the promoter.

One conclusion from studies with various types of activators is that many activators seem to function by helping recruit DNA-binding domains of RNAP to DNA, thus supplementing suboptimal RNAP–DNA interactions with protein–RNAP interactions. Apparently, most activators function by binding to target promoters before acting on RNAP. However, an alternative mechanism has been proposed for the MarA and SoxS regulators, in which they interact with free RNAP before binding to promoter DNA. In most cases, multiple activators bind independently at their target promoters. However, there are a few promoters at which activators bind cooperatively.

Small Ligands

Small ligands provide an alternative mechanism by which RNAP can respond quickly and efficiently to the environment. The best example is guanosine 3′,5′ bisphosphate, ppGpp, and also pppGpp, which are synthesized when amino acid availability is restricted to the extent that translation is also limited. Transcription of the seven transcription units that encode the rRNAs is mostly affected by the levels of ppGpp. A crystal structure shows that ppGpp binds near the catalytic centre of RNAP. This location might allow it to alter interactions with the incoming nucleoside triphosphates, with the catalytic magnesium and perhaps with the non-template DNA strand to destabilize open complexes. ppGpp has a co-regulator, the polymerase-binding DksA protein, which lowers the concentration of ppGpp needed to inhibit transcription. DskA exaggerates the effects of ppGpp by adapting the polymerase for regulation at promoters that are affected by the nucleotide. ppGpp also favors the association of other sigma factors with core polymerase at the expense of the σ^{70} holooenzyme that transcribes the ribosomal promoters. It has been proposed that ppGpp controls expression of the translation machinery in response to sudden starvation, whereas ATP availability controls expression in response to growth rate.

For obvious reasons, rRNA transcription is subject to complex regulation. Hence, in addition to the levels of ppGpp, the promoters of these genes respond to other factors, for example, they show an unusually high affinity for the first two nucleotides that form the 5′ end of the ribosomal transcript. This property, which can be explained by the unusual features of the ribosomal promoters, allows selective inhibition of rRNA transcription when nucleotide concentrations decrease, as is the case in stationary phase. The net amount of rRNA transcription is a function of the ratio of inhibitors to activators – H-NS/Fis (see section ‘Transcription factors’) and ppGpp/

NTP. Both of these ratios are usually highest during slow growth and lowest during rapid growth.

Small RNAs

A subset of small RNAs has been found to regulate transcription in bacteria. One example is the abundant 6S RNA that inhibits transcription at many σ^{70}-dependent promoters during stationary phase by binding to the active site of σ^{70}-RNAP and competing for DNA binding. The conserved secondary structure of 6S RNA, a single-stranded central bulge within a highly double-stranded molecule that is essential for 6S RNA function, has led to the proposal that 6S RNA mimics the open conformation of promoter DNA. Not only does 6S RNA block access to promoter DNA, but, surprisingly, it is used as a template for RNA synthesis. Synthesis of the templated RNA relieves the inhibitory effect of 6S RNA when cells encounter new nutrient sources and resume growth.

Regulation of Transcription Initiation via Changes in DNA Topology

The template for transcription is a negatively supercoiled DNA. Because the formation of an open transcription complex requires DNA melting, and because the degree of superhelicity affects the energy needed for the melting, it was anticipated that the superhelical character of a template would affect the properties of this template. Indeed, the efficiency of some promoters is influenced by the degree of supercoiling. Most of these promoters are stimulated by negative supercoiling, although few are inhibited. The effects of superhelicity on the process of transcription initiation have been shown *in vitro* in numerous studies and *in vivo* by the use of inhibitors of gyrase, which introduces negative supercoils. The reason why some promoters are sensitive to the degree of supercoiling, whereas others are not, might have to do with the fact that the sequence of some promoters is easier to melt and is therefore less dependent on supercoiling. Alternatively, because various regions on the bacterial chromosome are believed to have different degrees of supercoiling, the location of the promoter might determine whether it is sensitive to changes in superhelicity.

Transcription Elongation

The initiation phase ends when RNAP succeeds in extending the RNA chain beyond the first nine nucleotides and escapes from the promoter. At that stage the elongation process begins and the enzyme starts moving along the DNA, extending the growing RNA chain. During the transition from initiation to elongation, the size and shape of the RNAP undergoes successive changes. The first change is the loss of the σ factor. Whereas the holoenzyme covers approximately 75 bp (from -55 to $+20$), after the loss of σ, the polymerase covers approximately 55 bp (from -35 to $+20$). At that stage the polymerase is displaced from the promoter (promoter clearance or escape) and undergoes a further transition to form the elongation complex, which covers only 35–40 bp, depending on the stage during elongation. The polymerase now becomes tightly bound to both the nascent transcript and the DNA template, making it very stable.

The average rate of transcript elongation by the various RNAPs is 40 nt/s. However, this rate varies dramatically among RNAP and loosely correlates with the subunit complexity of the enzyme. Thus, the simple single-subunit bacteriophage RNAPs are the most rapid of all DNA-dependent RNAPs (several hundred nt/s), bacterial RNAPs transcribe at an intermediate rate (50–100 nt/s), and eukaryotic polymerases, although diversified, appear to be the slowest (20–30 nt/s).

Recent studies of both prokaryotic and eukaryotic transcription have yielded an increasing appreciation of the extent to which gene regulation is accomplished during the elongation phase of transcription. Nevertheless, RNAPs are not as accurate as DNA polymerases. The difference in fidelity between RNA and DNA polymerases is apparently due to the higher robustness of the proofreading mechanism that characterizes DNA polymerases than that of RNAPs. Of course, the fidelity of DNA replication is of greater importance than that of transcription because, unlike replication errors, misincorporation during transcription does not result in permanent and inherited genetic changes.

Blocks to Transcription Elongation

Transcript elongation does not occur at a constant rate. Throughout the elongation phase, RNAP can be paused, arrested, or terminated. These are important events that underlie many regulatory mechanisms that govern gene expression. During a ‘transcriptional pause’, the polymerase temporarily stops RNA synthesis for a certain amount of time, after which it can resume the elongation process. Thus, pausing can be described as transcriptional hesitation. In contrast, during a ‘transcriptional arrest’ the polymerase stops RNA synthesis and cannot resume it without the aid of accessory proteins. Throughout both pauses and arrests, RNAP remains stably bound to the DNA template and to the nascent transcript. These features distinguish paused and arrested polymerases from those that have terminated and thus detached from the DNA. Pausing and termination are sometimes related because pausing is a prerequisite for termination (see below). However, not all pauses are termination precursors. The time it takes a stalled polymerase to resume elongation varies among pause sites from very short periods of time, which cannot be accurately measured, to several minutes. The fraction of RNAP molecules that respond to an elongation block is also variable because ternary complexes differ in their ability to recognize pausing signals. Elongation is controlled by transcription factors, such as GreA, NusA, NusG and Mfd, that affect RNAP processivity by modulating transcription pausing, arrest, termination or antitermination.

Transcriptional pause and arrest signals can be intrinsic; that is, sequences in the nascent transcript or in the DNA template whose interaction with RNAP can inhibit the progression of the ternary complex, such as RNA regions, which have the propensity to form a stable secondary structure. In addition, extrinsic factors may obstruct the progress of RNAP during transcript elongation. There are numerous examples of RNAPs from various organisms being physically blocked by DNA-binding proteins during RNA synthesis in natural and artificial systems. An example is the purine repressor, which binds well downstream from the *purB* operon transcriptional start point and blocks the polymerase during elongation (it should be noted that in many cases RNAP is able to transcribe beyond the DNA-binding proteins in its path by either displacing them or bypassing them). In addition to DNA-binding proteins, which are the most obvious obstacles for RNAP, factors that perturb the structure of DNA can also inhibit the progression of RNAP and thus interfere with transcript elongation, for instance, extreme positive or negative supercoiling, unusual DNA structures such as Z-DNA, and DNA lesions. The efficiency of such potential impediments to block RNAP from elongating depends on various local factors and on the type of the RNAP. For example, T7 RNAP can efficiently bypass gaps in the DNA template strand that are 1–5 nt, and less efficiently gaps as large as 24 nt.

Pausing appears to occur by a two-tiered mechanism. An initial rearrangement of the RNAP active site interrupts elongation and puts the enzyme in an off-line state, called the elemental pause; additional rearrangements or interactions with regulatory proteins or downstream DNA sequences can create long-lived pauses. Transcriptional pausing is involved in various regulatory mechanisms. It plays a fundamental role in coupling transcription and translation by halting RNAP to allow a translating ribosome to catch up to polymerase. Given that most RNA exhibits significant secondary structure, even low-efficiency pausing that occurs with sufficient frequency will maintain coupling. Pause sites in the leader regions of operons regulated by attenuation are thought to halt RNAP to ensure that ribosomes are properly located to control the termination decision. The discovery of any attenuation mechanisms that are coupled with small molecule–RNA interactions (riboswitches) highlights the importance of pausing to proper regulation. Pausing also appears critical to ensure binding of elongation regulators to RNAP, for instance of RfaH to polymerase molecules paused at *ops* sites in the leader regions of RfaH-regulated operons. Furthermore, pausing plays a key role as the first step in both ρ-dependent termination and intrinsic termination of transcription (transcription termination is discussed in the section titled 'Transcription termination'). Pausing halts RNAP at terminators until ρ factor interacts with the transcription elongation complex. Additionally, specific pause sites have been found to play important roles in the proper folding of the nascent RNA. The combined effects of pausing in maintaining transcription–translocation coupling and in facilitating ρ-dependent termination when translation fails creates a prokaryotic version of an mRNA surveillance pathway, in which RNA damage or mutation that would lead to defective protein synthesis causes termination of transcription.

Although transcriptional arrest has been characterized *in vitro* and the evidence for its occurrence in the cell is only circumstantial, it is also believed to be implicated in the regulation of many genes. These predictions are based on the recognition that if an arrest occurs within the coding region of a gene, the arrested complex would block subsequently initiated RNAPs, thereby effectively repressing RNA synthesis from the affected gene.

Transcript Cleavage during Elongation

When RNAP encounters a roadblock during elongation, it backtracks and the 3′-end of the nascent RNA is cleaved to generate a new 3′-terminus. Transcript cleavage serves to rescue RNAPs that are arrested during elongation and gives the enzyme a second chance to transcribe over the roadblock and resume elongation. Following the cleavage, RNAP can correctly resynthesize the discarded RNA segment and continue with the elongation. The cleavage reaction involves accessory proteins in addition to RNAP. In *E. coli*, the GreA and GreB proteins serve as cleavage-stimulatory factors. GreA and GreB also affect the size of the released 3′-fragment. GreA-induced hydrolysis generates mostly di- and trinucleotides, while GreB-induced hydrolysis generates fragments of up to 18 nt long. GreA can only prevent transcription arrest, whereas GreB can reactivate prearrested transcription complexes as well.

Besides antipausing and antiarresting function, the factor-induced endonucleolytic reaction may enhance transcription fidelity by inducing the excision of misincorporated nucleotides, and by facilitating transition of RNAP from the initiation to the elongation stage of transcription. Molecular models for the mechanism of Gre proteins action were recently proposed based on biochemical, mutational, and structural analyses. Other factors, such as NusA, can regulate the cleavage properties induced by GreA and GreB. NusA is a multifunctional transcription factor that may elicit opposite effects on transcription elongation and termination. Its activity is discussed in the section titled 'Transcription termination'.

Another factor that functions in the cell to reactivate or recycle stalled or arrested RNAP during elongation is the transcription repair coupling factor Mfd. It does so by 'reverse backtracking' the RNAP, allowing its catalytic center to reengage the RNA 3′-end. Mfd also recruits DNA excision-repair machinery to damaged DNA sites in a transcription-coupled manner through its recognition of stalled RNAP molecules.

The Inchworm Model for Transcription Elongation

The old-fashioned view of the elongation process as a smooth forward motion during which RNAP moves 1 bp along the DNA template for every base added to the newly synthesized RNA chain might still hold for some regions of DNA. However, evidence has accumulated for a different type of movement of the polymerase during elongation. Thus, a new model for RNAP translocation has evolved: the 'inchworm model'. This model describes the movement of RNAP on the DNA template in a discontinuous inchworm-like fashion. The model predicts that the process of RNA chain elongation is a cyclic process that consists of discrete translocation cycles. Each cycle involves the steady compression of the RNAP on the DNA template followed by a sudden expansion. According to this model, the upstream (back) boundary of the enzyme moves steadily during elongation, as the RNA chain is extended. However, the downstream (front) boundary of the enzyme does not move while several nucleotides are added; it then 'jumps', that is, it moves 7–8 bp along the DNA. At the beginning of each cycle, RNAP stretches across ~35 nt of template DNA; it gradually compresses from the back end till it covers only ~27 nt; it then releases from the front end and stretches again to cover ~35 nt. As the RNAP compresses, the nascent RNA chain of the complex becomes longer and the single-stranded transcription bubble enlarges as well. The internal tension that these changes probably create in the enzyme is released when the front end expands discontinuously.

The inchworm model for transcription elongation postulates that RNAP binds to the DNA template at two separate sites, one downstream in the direction of transcription and one upstream, which can move independently of each other. This permits the polymerase to move in an inchworm-like manner, so one DNA-binding site on the polymerase remains fixed to the DNA, whereas the other moves along the DNA. There is now both direct and indirect evidence that validate this assumption. The downstream DNA-binding site in the *E. coli* polymerase was found to be double-strand-specific, whereas the upstream is single-strand-specific and interacts with the template strand. The model also assumes that the catalytic site of RNAP is linked to the movement of the upstream DNA site, but can move independently of the downstream DNA-binding site. The inchworm model also makes predictions about the existence of more than one RNA-binding site on the ternary complex. It has been shown that nascent RNAs interact with at least three sites on the *E. coli* polymerase, two on the β-subunit, and one on the β'-subunit. It is believed that together the DNA and RNA sites account for the remarkable stability and flexibility of ternary complexes. However, the precise size, placement, and strand specificity of these nucleic acid-binding sites are currently being elucidated.

Transcriptional Slippage

RNAP usually synthesizes RNA transcripts that are precisely complementary to the DNA template. However, in rare circumstances, RNAP can undergo transcriptional slippage that results in the synthesis of a transcript that is either longer or shorter than the sequence encoded by the DNA template. Such a slippage appears to occur when the polymerase transcribes homopolymeric runs. It has been proposed that the generation of transcripts that are shorter than the encoding template is due to translocation of RNAP without the incorporation of nucleotides, whereas the longer products are due to RNAP-incorporating nucleotides without translocation. Transcriptional slippage can occur during both the initiation and the elongation phases. However, the minimal length of the consecutive template nucleotides that can promote slippage in the two phases is different. During initiation, homopolymeric runs as short as 2 or 3 nt can be reiteratively transcribed by RNAP. During elongation, RNAP tends to slip only on longer runs, but the precise requirements have not been elucidated. In one case, slippage by the *E. coli* RNAP during elongation was reported to require runs of at least 10 dA or dT nucleotides, whereas runs of dG at the same length did not result in slippage. In some cases, the ability to slip seems to require a transcriptional pause in addition to the homopolymeric run. Transcriptional slippage is sometimes an important means of regulating transcription. It has been reported to play an important role in the regulation of transcription initiation at several bacterial operons, for example, *pyrBI*.

Implications of DNA Topology on Transcription Elongation

Because DNA has a helical secondary structure, a rotation about its axis is necessary to accomplish transcription elongation. This requires either that the entire transcription complex rotates about the DNA or that the DNA itself rotates about its helical axis. Under conditions in which the RNAP rotation is constrained, for example, due to the presence of ribosomes attached to the nascent RNA chain (which is often the case in bacteria), the DNA will rotate through the enzyme. Consequently, the process of transcription will tend to generate positive supercoils in the DNA ahead of the advancing RNAP and negative supercoils behind it. Excessive torsional stress in the DNA will arise if the DNA is anchored at various points (as is the case for circular DNA, such as the bacterial chromosome) or from the movement of RNAP in opposite directions along the DNA. DNA topoisomerases are the natural candidates to remove this tension. It was suggested that gyrase, which can relieve positive supercoils, and topoisomerase I, which removes negative supercoils, amend the situation in front of and behind the RNAP, respectively. This model is supported by the finding that when the activities of gyrase and topoisomerase I are inhibited or otherwise defective, transcription causes major changes in DNA supercoiling. A possible implication of this is that transcription, in addition to having a significant effect on the local structure of DNA, is responsible for generating a significant proportion of supercoiling that occurs in the cell.

Implications of DNA Replication on Transcription Elongation

Transcription regulation is carefully coordinated with DNA replication and chromosome segregation. In *E. coli* and in other bacteria and bacteriophages, heavily transcribed genes are oriented such that replication and transcription occur in the same direction. Despite this arrangement, because DNA replication occurs 10–20 times faster than transcription, RNAP and DNA polymerases do collide. The outcome of such an encounter is hard to predict. In *E. coli* there is evidence suggesting that the replication fork can displace the elongation complex. However, in bacteriophage T4, the movement of the replication apparatus does not seem to disrupt the elongation complexes, regardless of the direction of their motion relative to the replication fork. Interestingly, when direct collisions occur between the DNA and RNAPs of T4, the RNAP switches from the original DNA template strand to the newly synthesized daughter strand. The mechanism that allows the strand exchange without the dissociation of the elongation complex is not known, but probably relies on the various contacts with the DNA and RNA. Whatever the mechanism, the cell needs to coordinate the replication and transcription processes carefully.

Transcription Termination

Transcriptional elongation is highly processive and can lead to the production of RNA transcripts that are thousands of nucleotides long. The processivity is due to the high stability of the complex between the RNAP and the nucleic acids during elongation. It is this stability that necessitates the involvement of specific signals and factors to implement termination of transcription. To enable efficient termination, the termination signals or factors should cause drastic alterations of the interactions that are responsible for the stable elongation. At termination, RNAP stops adding nucleotides to the RNA chain, all the hydrogen bonds that hold the RNA–DNA hybrid together break leading to the release of the transcript, the DNA duplex reforms, and the enzyme dissociates from the DNA template. The sequence of these events is still not clear because attempts to determine whether the release of the RNAP is simultaneous with the transcript release or occurs subsequently have given ambiguous results. Once the transcript is released from the complex, it is unable to reattach in a way that allows transcriptional elongation to resume. Therefore, the transcript release is the commitment step that makes the termination process irreversible. On one hand, this mechanism ensures the termination at the end of genes and prevents the expression of adjacent distinct genetic units; on the other hand, this mechanism provides an opportunity to control gene expression.

The exact point at which termination of an RNA molecule occurs in the living cell is difficult to define. The 3′-end of an RNA transcript looks the same whether it is generated by termination or by cleavage of the primary transcript. Therefore, the best identification of termination sites is provided by systems in which RNAP terminates *in vitro*. An authentic 3′-end can be identified when the same end is generated *in vitro* and *in vivo*. In *E. coli*, two types of terminators were discovered, intrinsic terminators that do not require ancillary proteins and terminators that require the involvement of termination factors.

Intrinsic Terminators

Intrinsic terminators are sites at which core polymerase can terminate transcription *in vitro* in the absence of any other factor. The best characterized intrinsic terminators are the ones recognized by *E. coli* RNAP. Intrinsic terminators are characterized by a GC-rich sequence with an interrupted dyad symmetry followed by a run of about 6–8 dA residues on the template strand.

The transcription of the GC-rich sequence with the interrupted inverted repeats will give rise to an RNA segment that has the potential to fold into a stable stem-and-loop secondary structure (sometimes described as a hairpin structure). There is much indirect evidence that this structure is indeed formed in the nascent RNA. For example, mutations that interrupt the pairing in the stem part decrease the efficiency of termination, and compensatory mutations that restore the pairing recover the efficiency. There is also a strong correlation between the predicted stability of the structure and the termination efficiency. DNA oligonucleotides that are complementary to one arm of the stem in the stem-loop structure effectively reduce the efficiency of termination, presumably by annealing to the RNA sequence, and thus prevent the RNA from folding into the stem-loop structure. The sequence of the loop in the stem-loop structure also influences the stability of the RNA secondary structure, but the rules for contributing to loop stability have not been fully elucidated. How does the stem-loop structure contribute to termination? It is suggested that the formation of this structure in the newly synthesized RNA sequence, which is still in contact with the polymerase, causes the polymerase to pause, and thus destabilizes the ternary complex.

The other structural feature of an intrinsic terminator, the run of the dA residues (which is sometimes interrupted) in the template strand, is located at the very end of the transcription unit. The transcription of this sequence will generate a run of rU residues at the 3′-end of the RNA transcript. The hybrid between the dA and the rU residues is significantly less stable than most other hybrids, due to weak base-pairing, and it thus requires the least energy to break the association between the strands. This poor base-pairing is assumed to unwind the DNA–RNA hybrid and destabilize the interaction of the nucleic acids with the paused polymerase. The importance of the dA run has been established by mutational analysis. The importance of the length of the dA stretch was confirmed by introducing deletions that shortened this element; although the polymerase could still pause at the stem-loop, it no longer terminated. Interestingly, the actual termination can occur at any one of several positions toward the end of the dA run.

The DNA sequence within 30 bp downstream to the transcription stop point, which does not reveal an obvious consensus sequence, is also important for termination in certain cases. For example, changes in the sequence 3–5 bp downstream to the stop point of T7 early-gene terminator can reduce the efficiency of termination from 65 to 10%. Although these sequences are not transcribed, they are near or within the contact point between the RNAP and the DNA in the transcription complex. The way these sequences can affect transcription is by influencing the unwinding of the DNA or the progression of the polymerase along the DNA. Alternatively, the stability of the binding of the polymerase could vary depending on the sequence at the contact points.

Rho-Dependent Termination

The best characterized termination factor is the bacterial ρ protein. ρ is a classic termination factor in the sense that it provides a mechanism for dissociating nascent transcripts at sites that lack intrinsic terminators. Rho is essential for the survival of most bacteria, although in some prokaryotes Rho is dispensable, or absent altogether. Rho binds to the nascent RNA chain. The sequences that form a ρ-dependent terminator extend from at least 60 bp upstream to about 20 bp downstream of the actual stop point. ρ-binding sites show a highly inconsistent sequence homology and their only common feature is a relatively high cytosine content. In addition, ρ has a strong preference for sufficiently long segments of unstructured RNA (lacking base-pairing).

ρ causes RNAP to terminate preferentially at points that are natural pause sites. These pause signals often encode U-rich RNA–DNA hybrids, which have the added consequence of destabilizing the transcription elongation complex. There is no evidence that ρ affects the elongation-pausing specificity of the polymerase, but several lines of evidence point to a highly specific conformation of the elongation complex preferred by ρ. In addition to ρ being an RNA-binding protein, ρ contains an RNA–DNA helicase activity; it hydrolyzes ATP to energize the separation of an RNA–DNA hybrid. Thus, ρ is acting primarily as an RNA-release factor. The recently solved crystal structure of ρ could explain many of its activities, although many questions pertaining to ρ-recognition sites, enzymatic activities, and interactions with the elongation complex remain obscure. The current model for ρ action is that it binds to the RNA transcript at sites that are unstructured and rich in C residues; it then translocates along the RNA until it catches up with the polymerase at sites where the enzyme pauses; ρ unwinds the RNA–DNA hybrid in the transcription bubble; termination is completed by the release of ρ and RNAP from the nucleic acids. Some ρ mutations can be suppressed by mutations in the genes that encode the β- and β'-subunits of RNAP, implying that in addition to interacting with the nascent RNA chain, ρ also interacts with the polymerase.

The lack of stringent sequence requirements for a ρ-dependent transcription terminator raises the possibility that such terminators might be fairly frequent in DNA sequences, not only at the ends of operons but also within genes. What prevents ρ from terminating within genes? Because transcription and translation are coupled in prokaryotes, the mRNA chain that emerges from the transcription complex is protected by ribosomes, probably preventing ρ from gaining access to the RNA. The phenomenon of polarity (a nonsense mutation in one gene prevents the expression of subsequent genes in the operon) can be explained by the release of ribosomes from the transcript at the nonsense-mutation site, so that ρ is free to attach to and move along the mRNA; when it catches up with RNAP, it terminates transcription, thus preventing the expression of distal parts of the transcription unit. What prevents ρ from acting on transcripts that are not translated, such as rRNAs and tRNAs? One reason seems to be the lack of ρ-binding sites on these RNAs because they are highly structured. rRNA molecules are further protected by the binding of ribosomal proteins. Another mechanism that protects rRNA operons against ρ-dependent termination relies on sequences near the start of the rRNA genes that dictate antitermination. It was suggested that this mechanism increases the rate of transcriptional elongation of rRNA operons by preventing pausing.

Auxiliary Termination Factors

Although some polymerases can spontaneously terminate transcription at intrinsic terminators, the efficiency of termination *in vitro* is often enhanced significantly by the presence of additional factors. It therefore seems that the DNA signals that characterize intrinsic terminators are necessary, but sometimes not sufficient. The best characterized of these auxiliary termination proteins is NusA. A less-studied factor named τ (tau) is known to enhance and modify recognition of some strong intrinsic terminators for *E. coli* RNAP. ρ-dependent termination can also be enhanced by an auxiliary factor, the NusG protein. Small RNAs were also shown to affect transcription termination via base-pairing interactions with sequences in the mRNA.

The ability of NusA to increase the efficiency of termination at some intrinsic terminators might be attributed to its capability to increase the rate of pausing at certain sites. Enhanced pausing would allow more time for the conformational change that leads to the release of the nascent transcript. Some intrinsic terminators that are predicted to form not a very stable secondary structure, such as the one in the ribosomal protein S10 operon leader, depend on NusA for their operation, and can therefore be defined as NusA-dependent terminators. In the case of the intrinsic terminator in the attenuator region preceding the gene for the β-subunit of *E. coli* RNAP, NusA, rather than enhancing termination, reduces termination efficiency. Indeed, as mentioned before, NusA may elicit opposite effects on transcription, depending on the RNA–DNA sequence context and the presence or absence of auxiliary factors. By itself, NusA stimulates certain types of pausing and ρ-independent intrinsic transcription termination. However, NusA can induce anti-termination at ρ-dependent terminators with RNA sequences containing ‘nut’ or ‘nut’-like elements (see below). In complex with other Nus factors (NusG, NusB, NusE) or λ phage proteins N and Q, it stimulates antitermination at both ρ-dependent and ρ-independent terminators (see below). NusA antitermination function plays an important role in the expression of ribosomal genes. During transcription of many other genes, NusA-induced RNAP pausing provides a mechanism for synchronizing transcription and translation.

NusG has a minor effect on termination at some intrinsic terminators. However, it plays a significant role in the functioning of some ρ-dependent terminators. This role was deduced from the strong effect of NusG loss on the activity of some ρ-dependent terminators *in vivo* and from the effect of NusG on ρ termination efficiency and pattern across a terminator in a purified *in vitro* system. The role of NusG in ρ-dependent termination is not conclusive, but the evidence hints that NusG might increase the retention of ρ on the nascent transcript, thereby increasing its local concentration, and/or that NusG increases the rate of RNA release at termination sites. It is also possible that NusG acts directly to enhance ρ activity or indirectly by slowing the dissociation of ribosomes from the RNA, thus preventing ρ from binding to the transcript.

Two proteins that antagonize ρ-dependent transcription termination have been identified: the Psu protein encoded by bacteriophage T4 and YaeO from *E. coli*. Psu seems to inhibit ρ-dependent termination by slowing down the translocation of ρ along the RNA, as this protein was shown to negatively affect the ATPase activity of ρ. A model of the YaeO–ρ complex, which is based on the solved structure of the two proteins, proposes that YaeO binds to ρ, acting as a competitive inhibitor of RNA binding.

Interestingly, the presence of the σ factor in excess significantly increases the rate of RNAP recycling. Hence, the σ factor can also be considered a termination factor. This activity supports the notion that σ can remain associated with the polymerase at postinitiation steps.

Antitermination

Antitermination is used as a control mechanism in phages to regulate the progression from one stage of gene expression to the next, and in bacteria to regulate expression of some operons. Antitermination occurs when RNAP reads through a terminator into the genes lying beyond. The terminators that are bypassed can therefore be defined as conditional terminators. Antitermination is not a general mechanism that can occur in all terminators, but is, rather, dependent on the recognition of specific sites in the nucleic acids. Many mechanisms involve choosing between two alternative hairpin structures in an RNA transcript, with the decision dependent on interactions between ribosome and transcript, tRNA and transcript, or protein and transcript. In other examples, modification of the transcription elongation complex is crucial to make it bypass certain terminators.

The N protein of bacteriophage λ mediates antitermination necessary to allow RNAP to read through the terminators located at the end of the immediate early genes in order to express the delayed early genes. The recognition site needed for antitermination by N, termed *nut* (for N utilization), lies upstream from the terminator at which the action is eventually accomplished. The *nut* site consists of two sequence elements, a conserved 9-nt sequence called BoxA, which is also an antiterminator signal in the operons encoding ribosomal transcripts, and a 15-nt sequence called BoxB, which encodes an RNA that would form a short stem structure with an A-rich loop. A number of host proteins, including NusA, NusG, ribosomal protein S10 (NusE), and NusB, participate in the N-mediated antitermination process (Nus stands for N utilization substance). A model for N-mediated antitermination at ρ-dependent terminators proposes that N recognizes and binds to the BoxB stem-loop structure formed on the nascent transcript, whereas NusB and S10 bind to the BoxA sequence on the RNA. These proteins are held together through interactions with core RNAP that are stabilized by NusA and NusG. Hence, a ribonucleoprotein complex is formed at the *nut* site and stays attached to the elongating RNAP. This complex prevents RNAP from pausing, thus denying the ρ factor the opportunity to cause termination, and the polymerase continues past the terminator. N also suppresses termination at intrinsic terminators; however, NusA suffices for N to prevent termination at these sites. Other phage related to λ have different N proteins and different antitermination specificities. Each phage has a characteristic *nut* site recognized specifically by its N-like protein. All these N-like proteins seem to have the same general ability to interact with the transcription apparatus in an antitermination capacity.

The Q protein is required later in bacteriophage λ infection. It allows RNAP to read through the terminators located at the end of the immediate early genes, to express the late genes of bacteriophage λ. Q has a different mode of action than N. It recognizes and binds to a site on the DNA, called *qut*. The upstream part of *qut* lies within the λ-promoter P_R, whereas the downstream part lies at the beginning of the transcribed region. Thus, Q antitermination is specific for RNAP molecules that have initiated at the P_R promoter. The part of *qut* that lies within the transcribed region includes a signal that causes RNAP to pause just after initiation. This pause apparently allows Q to interact with the polymerase. Once bound, the Q-modified enzyme is released from the pause and is able to read through most transcription terminators, both intrinsic and ρ-dependent. It seems that the modification of the polymerase by Q increases the overall rate of transcription elongation and permits the polymerase to hurry past the terminators. Interestingly, the pause of the polymerase early in the transcription unit, which is a prerequisite for Q-mediated antitermination, involves the binding of the σ subunit of the RNAP holoenzyme to the nontemplate strand of DNA in the transcription bubble up to 15 nt downstream from the start point of transcription. Thus, an initiation factor acts in concert with a DNA-binding termination factor to modify the elongation properties of RNAP. Once again, it is shown that σ can remain associated with the polymerase and play a role in postinitiation steps.

RNAP molecules that are engaged in transcribing the rRNA (*rrn*) operons are modified in a way that makes them bypass certain terminators within the rRNA genes. The modification is established by the recognition of a sequence signal that is nearly identical to the BoxA sequence involved in N-mediated antitermination. It has been shown that a heterodimer of NusB and S10 protein binds to the BoxA sequence on the RNA of one of the *rrn* operon. It was therefore proposed that the mechanism of transcriptional antitermination in the *rrn* operons, similar to the mechanism mediated by N, involves formation of a ribonucleoprotein complex on the BoxA complex that is carried along with the elongating polymerase. The probable purpose of this mechanism is to ensure that transcription of the rRNAs is immune from ρ action.

Some bacterial proteins regulate gene expression by binding to specific RNA sequences and alter the structure of the leader RNA to promote or prevent transcription termination. The TRAP protein from *B. subtilis* and the BglG protein from *E. coli* represent two families of proteins that promote termination and antitermination, respectively. The BglG protein, encoded by the β-glucoside utilization operon (*bgl)* in *E. coli*, prevents the termination of transcription at two intrinsic terminators. The first terminator is in the 5′ untranslated leader of the *bgl* transcript and the second is in the intergenic region between the first and second genes of the operon. BglG is an RNA-binding protein that recognizes and binds to a specific sequence partially overlapping the sequence of both terminators. By binding to its RNA target site, BglG stabilizes a secondary structure, which is an alternative to the terminator structure. Thus, BglG binding to the *bgl* transcript prevents the formation of the terminators and the polymerase can read through them. BglG exerts its effect as a transcriptional antiterminator only when the expression of the operon is required, that is, when β-glucosides are present in the growth medium. The activity of BglG as an antiterminator depends on its phosphorylation state, which affects its oligomeric form. BglG-like antiterminators were shown to control the expression of sugar utilization genes in various organisms, both Gram-negative and Gram-positive.

Unlike BglG, the TRAP protein from *B. subtilis* promotes termination of *trp* operon transcription. The transcript of the leader region of the *trp* operon can fold to form mutually exclusive antiterminator and terminator structures. When the TRAP protein is activated by tryptophan, it binds to the 5′ segment of the antiterminator hairpin, freeing its 3′ segment to pair with the adjacent 3′ RNA segment to form a terminator structure. Since the stability of the antiterminator is higher than that of the terminator, TRAP binding is required to prevent antiterminator formation. Thus, when cells have adequate levels of tryptophan, activated TRAP binds to the antiterminator region, the terminator formed, and transcription is terminated. When the level of tryptophan drops, an anti-TRAP protein is synthesized. This protein binds to tryptophan-activated TRAP and inhibits its ability to bind to *trp* leader RNA; this results in TRAP inactivation, leading to increased expression of all the genes required for tryptophan biosynthesis.

Transcriptional regulation of operons that are concerned with amino acid synthesis and utilization by ribosome-mediated transcription termination and tRNA-mediated transcription antitermination are described in a different article.

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Transduction: Host DNA Transfer by Bacteriophages

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Defining Statement
Introduction
Specialized Transduction
Generalized Transduction
Variations on Transduction
Transduction as a Genetic Tool
Transduction in the Environment
Conclusion
Further Reading

Glossary

abortive transductant A bacterium that has acquired transduced DNA, which has not been degraded or stably integrated into the bacterial DNA. Abortive transductant DNA can be expressed, but cannot be replicated.

gene transfer agent A prophage-like element that promotes generalized transduction of bacterial DNA but cannot replicate to form infective phage particles.

generalized transduction The phage-mediated transfer of any region of bacterial DNA from one bacterium to another. Generalized transduction can be mediated by temperate or virulent phage.

lysogen A bacterium that harbors a prophage.

prophage A phage in lysogeny. Prophage are replicated as part of the bacterial chromosome or as a plasmid-like element.

specialized transduction The phage-mediated transfer of regions of bacterial DNA, located adjacent to the site of prophage insertion, from one bacterium to another. Specialized transduction is mediated by temperate phage upon the incorrect excision of the chromosomal prophage.

temperate phage A phage that is capable of entering either the lytic or lysogenic life cycles.

transducing particle A phage capsid that has packaged bacterial DNA and hence is proficient for transduction.

transducing phage A phage capable of mediating transduction.

transductant A recipient bacterium that has stably acquired the transduced DNA.

transduction The phage-mediated transfer of bacterial DNA from one bacterium to another.

virulent phage A phage that is able to replicate only via the lytic cycle.

Abbreviations

***cos* sites**	cohesive end site
GTA	gene transfer agent
HFT	high-frequency transducing
HGT	horizontal gene transfer
HT	High-transducing
TEM	transmission electron microscopy

Defining Statement

Transduction is the bacteriophage-mediated transfer of host DNA between bacterial cells. Transduction occurs in the natural environment, where phage are numerous, and is predicted to be a significant driver of bacterial evolution. Model phage–host studies have revealed transduction mechanisms and have led to the development of sophisticated genetic methods based on transducing phage.

Introduction

Bacteria can acquire genes by vertical gene transmission to daughter cells and by horizontal gene transfer (HGT). The three main classes of HGT are transformation, conjugation, and transduction. This article will focus on transduction, with transformation and conjugation being dealt with in other chapters. Transduction is the bacteriophage (phage)-mediated transfer of bacterial DNA from a donor bacterium to a recipient bacterium. It was first observed by Zinder and Lederberg in 1952 whilst studying genetic recombination in *Salmonella enterica* serovar Typhimurium. The recombination observed did not require bacterial cell–cell contact and was not susceptible to DNase treatment, suggesting a genetic transfer mechanism distinct from conjugation and transformation. The temperate phage P22 was the agent responsible, and these authors coined the term transduction to describe this new form of gene transfer.

Transduction can be divided into two major types called specialized (or restricted) and generalized, which will be detailed in sections 'Specialized transduction' and 'Generalized transduction', respectively. Briefly, specialized transduction occurs when the prophage of a temperate phage incorrectly excises from the chromosome of the lysogen taking with it genes flanking the prophage insertion site. The recipient bacterium will acquire the 'new' genes when it is lysogenized by the specialized transducing phage or following recombination between homologous sequences in the transducing phage DNA and the recipient chromosome. In generalized transduction, which can occur during the lytic mode of phage growth of both virulent and temperate phage, segments of bacterial DNA roughly equal to the genome size of the transducing phage are accidentally packaged into capsids. The resulting transducing particles can still adsorb to, and inject DNA into, recipient bacteria and the transduced DNA may be incorporated into the recipient chromosome by homologous recombination, resulting in stable bacterial transductants. Variations on specialized and generalized transduction, including the effects of nonreplicative prophage-like gene transfer agents (GTAs), will be addressed in section 'Variations on transduction'.

Transduction, especially generalized transduction, has become a powerful tool in bacterial genetics. Phage have been used for gene mapping, construction of strains, and localized mutagenesis, etc. In the section titled 'Transduction as a genetic tool', we will discuss techniques that have utilized transduction and how transducing phage can be used as 'molecular reagents'. Transduction is a useful tool under laboratory conditions and also occurs in the natural environment. The formation of transducing particles is often considered an accidental process. However, evidence from bioinformatics and experiments on transduction in the environment suggest that transduction can benefit the bacterial hosts, and, therefore, their molecular parasites – the phage (see 'Transduction in the environment').

Specialized Transduction

Definition and Discovery

In 1956, a few years after the discovery of generalized transduction, the process of specialized transduction was first observed using *Escherichia coli* and phage lambda (λ). Since then, many other temperate phage that infect various bacteria have been shown to be proficient at specialized transduction. The term 'specialized' was derived from the ability of λ to mediate the transfer of only a limited number of *E. coli* genes. The main difference between specialized and generalized transduction lies in what DNA is packaged into the phage capsid. Whereas generalized transducers can package bacterial DNA without phage DNA, specialized transducers can package host DNA along with some, or all, of their own genome. Briefly, a specialized transducing particle arises from the incorrect excision of a prophage from the host chromosome and the resultant packaging of bacterial and phage DNA.

Bacteriophage λ is one of the most thoroughly studied and understood biological entities on the planet and our understanding of many of the known gene regulation mechanisms derives from ingenious experiments using this model phage. Not surprisingly, the most complete picture of the steps involved in specialized transduction has been elucidated using λ. Therefore, λ will be discussed to illustrate the general mechanism of specialized transduction.

Replication of Phage λ

To understand fully how specialized transduction occurs, it is necessary to discuss the process of λ replication. Lambda is the classic example of a temperate phage, being able to undergo two alternative life cycles: lytic and lysogenic. As mentioned earlier, specialized transduction relies on temperate phage that are able to form prophage during the lysogenic cycle. Therefore, the life cycle of λ, with the emphasis on the lysogenic process, will be outlined here and is depicted in **Figure 1**.

Phage λ recognizes host cells via the LamB outer membrane protein and, upon binding to this receptor, injects its linear double-stranded DNA (dsDNA) into the cell. The linear dsDNA then cyclizes via the pairing of *cos* sites (cohesive end site). The *cos* sites contain 12 nucleotide single-stranded overhangs that self-anneal via complementary base pairing and are covalently linked by ligation. Based on a well-characterized regulatory mechanism, the circular λ genome now 'decides' which life cycle to enter. Replication via the lytic cycle is promoted by the Cro regulatory protein, which results in the assembly of new phage particles and their release from the cell following bacterial lysis. Alternatively, during lysogeny, λ can integrate into the host chromosome and thus replicate as a prophage.

The initial insertion into the bacterial genome requires the phage attachment site (*attP*) on λ and the bacterial attachment site (*attB*) located between the galactose (*gal*) and biotin (*bio*) operons in *E. coli* (**Figure 1**). This insertion

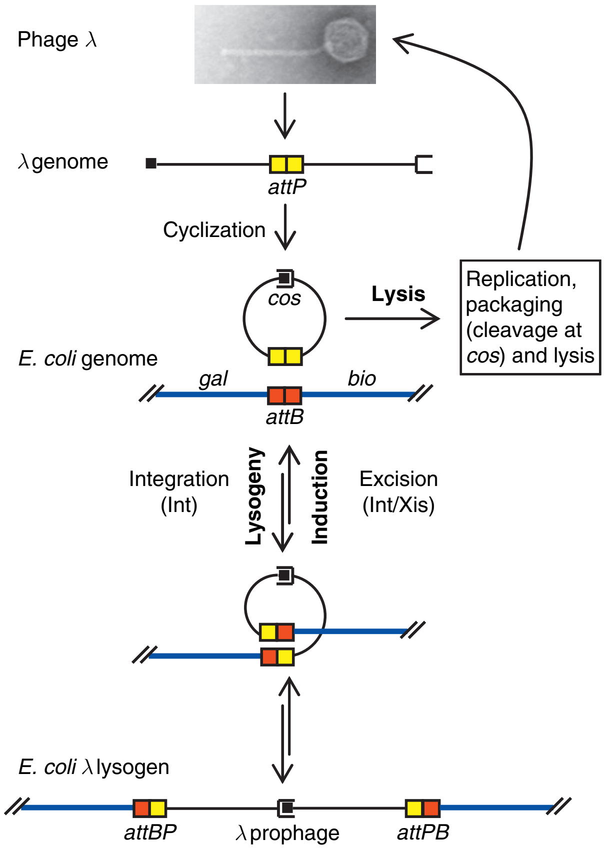

Figure 1 Normal phage λ lysogenic and lytic life cycles. The linear dsDNA genome of λ is injected into *Escherichia coli* following binding of the phage to the LamB receptor. The genome then cyclizes via the complementary single-stranded regions of the *cos* sites and is covalently closed by ligation. Lambda may then enter the lytic pathway with replication, packaging, and lysis of new phage particles from the cell. Alternatively, site-specific recombination between the λ *attP* sites and the *E. coli attB* site is promoted by Int, resulting in establishment of the λ prophage in the *E. coli* genome. Induction of the prophage by stresses (e.g., UV light) causes the excision of the λ prophage catalyzed by the Int and Xis proteins that recombine the hybrid *attBP* and *attPB* sites. The recyclized genome is then replicated, packaged, and released from the cell as mature λ phage particles. *E. coli* DNA is depicted in blue and phage λ DNA in black. Transmission electron microscopy (TEM) image of phage λ stained with phosphotungstic acid.

is catalyzed by the phage Int protein, a site-specific recombinase, that recognizes and promotes recombination between these short, relatively dissimilar, sequences. Int is a member of the tyrosine family of recombinases that have an active site tyrosine residue, which forms a covalent link to the 3′ phosphate after cleavage. Because λ is circular and integration occurs at *attP* and not the *cos* site, site-specific recombination into the bacterial chromosome results in a different linear gene order in the prophage than in the mature capsids. Once in the chromosome, the prophage state is maintained by the CI repressor and can be stably propagated upon bacterial DNA replication and cell division of these λ lysogens. However, factors such as cellular stress can trigger the regulatory switch from lysogeny to the lytic pathway, which will ultimately generate mature phage. The first step in this process requires the excision of the prophage to regenerate the circular form. Since the *attP* and *attB* sites are now hybrid sequences (**Figure 1**), they are not recognized by Int alone, and in addition require a phage excisionase called Xis. The combined action of Int and Xis catalyzes the site-specific recombination of the hybrid *att* sites and enables excision of the λ prophage, leading to the formation of the circular λ genome. Lambda then begins replication to generate long concatemers of λ DNA interspersed with *cos* sites. Once phage heads are assembled, the DNA is packaged and cleaved into genome length units in a single-stranded staggered manner, regenerating each of the *cos* sites at the ends of the linear dsDNA. This process of packaging involves numerous phage and host proteins. Phage tail proteins are then added to the heads and the completed phage particles are released from the host following lysis and can reinitiate another infective cycle.

Incorrect Prophage Excision Creates Specialized Transducing Particles

Occasionally, the λ prophage does not excise using the Int/Xis system, but instead recombines in a nonspecific manner (illegitimate recombination) as shown in **Figure 2**. These excision events are rare (approximately 10^{-6} compared with normal excision) and result in circular phage DNA molecules that contain phage and bacterial genes. If the site of illegitimate recombination maintains the approximate size of the λ genome and still contains the *cos* sites, following rolling circle replication, these specialized transducing phage can be packaged into capsids. Due to the location of the λ prophage in the *E. coli* chromosome, the first genes shown to be transduced were the *gal* and *bio* operons that flank the insertion site. A number of methods have been developed to use λ to transduce genes that are not located adjacent to *attB*. Initially, strains that contained large deletions or rearrangements were utilized because they positioned genes closer to *attB* and enabled packaging by λ. In another strategy, the *attB* site was deleted and λ lysogens were isolated where the prophage had inserted with reduced specificity into the genome at alternative (secondary) sites. This allowed the transduction of genes located near these integration points. Furthermore, these experiments provided information about the sequence specificity and efficiency of the Int protein. Finally, it is possible to engineer λ to contain a transposon and then use the same transposon to mutagenize the bacterial host. The prophage is then able to insert into these sites in a process requiring homologous recombination. Therefore,

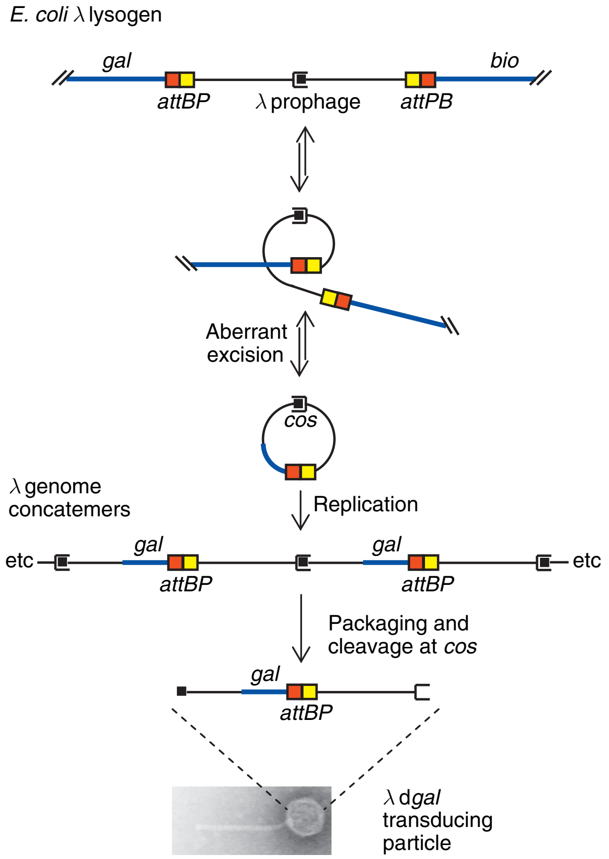

Figure 2 Aberrant excision of a λ prophage creates specialized transducing particles. At a low frequency, illegitimate recombination events cause incorrect excision of the λ prophage. This can result in the cyclization of phage genomes that lack some genes but, that now also carry *E. coli* chromosomal segments that were flanking the prophage. In the figure, the generation of a *gal* transducing particle is depicted. Replication of the genome produces a long concatemer that is packaged and cleaved at the *cos* sites resulting, in this example, in *gal* transducing phage particles. *E. coli* DNA is depicted in blue and phage λ DNA in black. Transmission electron microscopy (TEM) image of phage λ stained with phosphotungstic acid.

using this technique it is theoretically possible to insert λ in any nonessential gene in the *E. coli* genome and thereby transduce flanking host genes. Once the specialized transducing particles have been packaged they are capable of initiating infection of a new recipient bacterium.

Formation of Transductants

The dsDNA of the infecting transducing particle is injected into the new host and cyclized as described above. Because specialized transducing particles usually have lost some phage genes, this can have deleterious effects on λ function, especially in terms of integration and generation of new particles. For example, a *gal* transducing phage, λd*gal* (the 'd' means *d*efective), has lost the phage head and tail genes and, therefore, cannot undergo fully productive lytic growth, but can lysogenize the host. Alternatively, a *bio* transducer, λp*bio* (the 'p' means *p*laque forming), lacks the *int* and *xis* genes and subsequently is unable to become a prophage, but can replicate lytically.

To form a transductant, the horizontally acquired DNA must be maintained stably in the recipient cell via one of a number of possible routes. Homologous recombination between sequences common to both the transducer and host genomes can provide stable transductants that have incorporated the transducing phage genome (**Figure 3**(**a**)). These lineages will have two copies of the transduced DNA segment (merodiploid) and can be useful for complementation analyses. However, if multiple recombination events occur, the host locus may be exchanged for the equivalent transduced DNA (**Figure 3**(**a**)). This process of forming a haploid transductant is less frequent and is essentially how transductants are obtained by generalized transduction. Alternatively, introduction of the transduced DNA can proceed via the normal λ integration route, with the aid of a helper phage that provides the missing functions *in trans* or *in cis* via recombination (**Figure 3**(**b**)). First, coinfection with a wild-type λ and the transducing particle can result in recombination at sites shared by these phage, which can then integrate at *attB* via the *attP* and Int functions supplied by the helper phage. The resulting lysogens harbor the genomes of both the transducer and helper phage and hence are called double lysogens (or dilysogens). These dilysogens are extremely useful for the generation of high-frequency transducing (HFT) lysates upon prophage induction. A single excision of both prophage yields a hybrid circular DNA that is replicated into concatemers and packaged according to the *cos* sites, giving rise to alternate transducing and wild-type λ particles. The final mode of stable transductant formation can occur when λ is able to replicate as a plasmid in the recipient. Mutation of the λ *N* gene or the host *nusA* gene causes reduced expression of the λ *O* and *P* genes, which are required for replication and, at low levels, allows maintenance of λ essentially as a plasmid. Alternatively, acquisition of a plasmid origin of replication also provides λ DNA with stable replication and maintenance. Therefore, any one of these mechanisms can produce heritable transductants.

Other Specialized Transducing Phage

The λ phage has provided an excellent system for unraveling the mechanisms of specialized transduction. This model predicts that aberrant excision of any prophage that packages its DNA based on phage-specific sequences (analogous to *cos* sites) could lead to production of specialized transducing particles. Indeed, many specialized transducers have been identified, including *Pseudomonas*

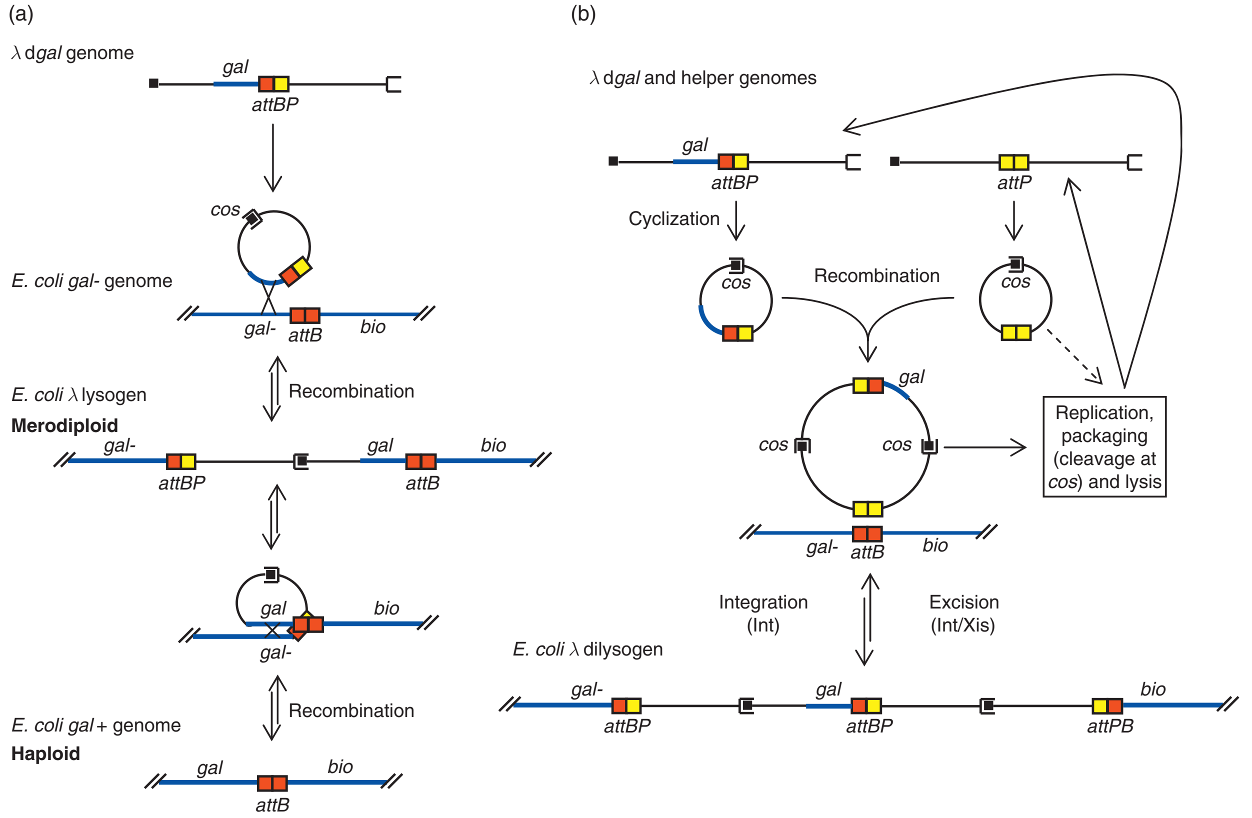
(a)
λ dgal genome
gal
attBP
cos
E. coli gal- genome
gal-
attB
bio
Recombination
E. coli λ lysogen
Merodiploid
gal-
attBP
gal
attB
bio
gal
bio
gal-
Recombination
E. coli gal+ genome
Haploid
gal
attB
bio
(b)
λ dgal and helper genomes
gal
attBP
attP
Cyclization
cos
Recombination
cos
gal
cos
cos
Replication, packaging (cleavage at cos) and lysis
gal-
attB
bio
Integration (Int)
Excision (Int/Xis)
E. coli λ dilysogen
gal-
attBP
gal
attBP
attPB
bio

Figure 3 Formation of transductants and the creation of high-frequency transducing lysates. (a) Formation of diploid and haploid transductants via Rec-dependent recombination with the chromosome of an *Escherichia coli gal* mutant. In this example, the λ*dgal* phage is used for illustrative purposes. A single recombination event between the shared *E. coli* sequences can result in the merodiploid lysogen shown, or one where the mutant *gal* allele is present in the prophage (not shown). A second recombination event that can lead to the replacement of the mutant *gal* allele with the wild-type *gal* sequence is possible. (b) Production of dilysogens occurs when an *E. coli gal* mutant is coinfected with wild-type λ and the specialized transducing particle (λ*dgal*). The phage genomes cyclize and can then recombine together at shared sequences and integrate into the host chromosome in an Int-dependent manner giving the dilysogen shown. Induction of the prophage leads to the excision of either the wild-type λ or the entire dilysogen, which are then capable of replication, packaging, and release from the cell. *E. coli* DNA is depicted in blue and phage λ DNA in black.

aeruginosa phage D3 and *Bacillus subtilis* phage SPβ. Comparative genomics may provide further numerical data on the frequency of specialized transduction since it is also theoretically possible that some of the apparently degenerate prophage present in the many sequenced bacterial genomes may have been the result of past specialized transduction events. Alternatively, there is evidence that mutational events leading to inactivation of prophage can occur following prophage acquisition by the host bacterium.

Generalized Transduction

Generalized transduction is the process whereby any section of the bacterial DNA can be transferred from one bacterium to another via a phage virion. This phenomenon was first identified by Zinder and Lederberg in 1952 in *Salmonella*, where the temperate phage P22 transferred chromosomal DNA from one strain of *Salmonella* to another. A few years later, in 1955, Lennox identified phage P1 as a generalized transducing phage of *E. coli*, and the knowledge about transducing phage and the mechanisms of generalized transduction has been gleaned from investigations using these two 'model' phage. Early studies of P22 transduction in *Salmonella* and P1 transduction in *E. coli* have been extensively reviewed elsewhere (see 'Further reading'). The findings from many of these studies, together with studies of other transducing phage, have contributed to current knowledge on generalized transduction, which is summarized below.

Properties of Generalized Transducing Phage

The known, naturally occurring generalized transducing phage are dsDNA tailed phage that utilize a sequential headful DNA packaging mechanism. Generalized transducing phage can be either virulent or temperate and occasionally, bacterial DNA, instead of phage DNA, is packaged into the head of an otherwise unaltered virion, resulting in a transducing particle instead of a fully functional phage. In order for this to occur, a generalized transducing phage must not degrade the host DNA completely upon infection. The life cycle of a generalized transducing phage is summarized in **Figure 4**.

Generalized transducing particles arise through a mistake in DNA packaging so that the host chromosomal or plasmid DNA is taken up into the phage head in place of the phage DNA. The frequency of this mispackaging, and therefore the frequency of transducing particle formation, is dependent upon the mechanism of DNA packaging employed by the phage.

During the phage lytic cycle, the structural proteins are made and the phage capsid proteins make up the virion head or prohead with the tails assembled separately. The DNA is then replicated and forms concatemers of phage genomes, arranged head to tail in tandem, usually around four genomes long, depending on the phage. This DNA must then be packaged into the proheads. In both P1 and P22, headful packaging is initiated by recognition of a specific sequence present in the phage DNA called the *pac* site by the phage packaging apparatus. The phage terminase recognizes the *pac* site and the DNA is cleaved at or near this site. The cleaved *pac* end is bound to the large terminase subunit, attached to the prohead, which initiates the packaging of the linear DNA concatemer into the prohead. The size of the head determines how much DNA can be packaged; hence the term 'headful packaging'. This is usually a little more than the size of the phage genome. For example, the genome of P1 is 95 kb in length, but up to 115 kb of DNA is usually packaged into the phage head. Therefore, in addition to a single copy of the phage genome, there is also some extra phage DNA packaged, which is terminally redundant. Once the prohead is full, the DNA is cleaved and the cut end of the remaining concatemer is recognized by the packaging apparatus and used to initiate packaging of the next empty prohead. Several proheads are filled sequentially in this manner from the remaining DNA,

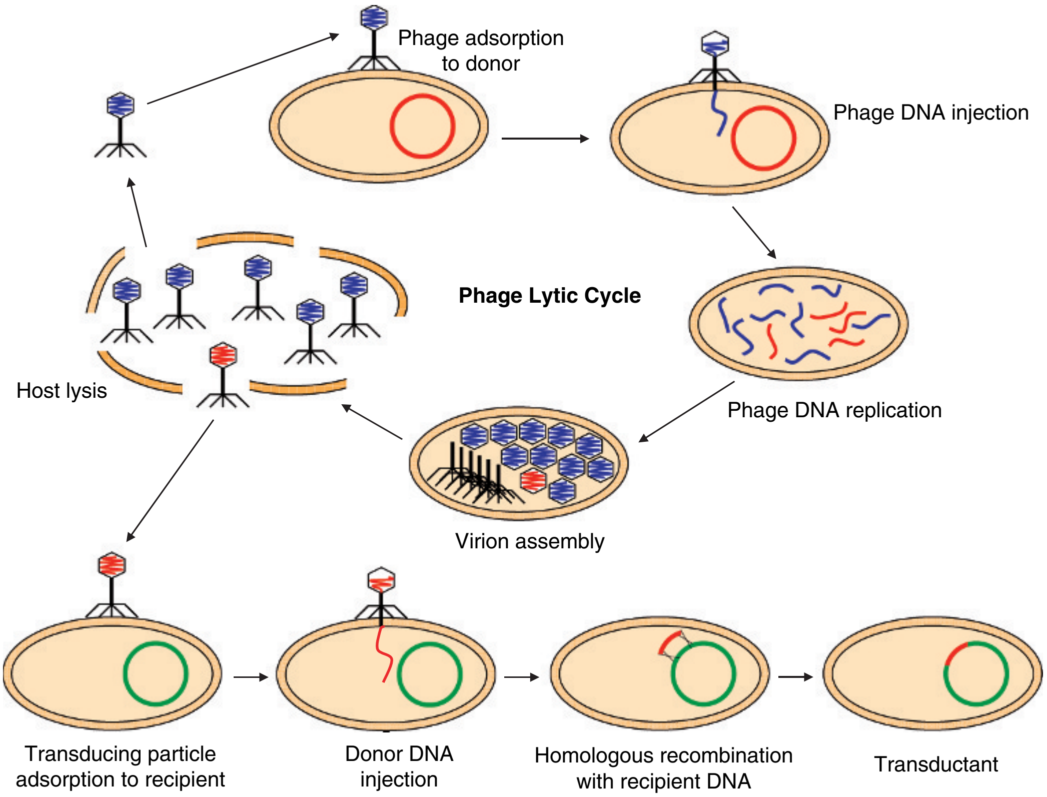

Figure 4 Generalized transducing phage life cycle. During the lytic cycle a phage adsorbs to a host bacterium, injects and replicates its DNA, makes virion heads and tails, packages its DNA into the heads, attaches the tails and lyses the host, releasing the phage to infect new bacteria. Upon infection by a generalized transducing phage, phage-encoded enzymes can cleave the bacterial DNA into large sections, and occasionally these lengths of bacterial DNA can be mispackaged into a phage head. A small number of virions made will contain host DNA in place of phage DNA, producing a transducing particle instead of a functional phage. Transducing particles can adsorb to, and inject the bacterial DNA into, susceptible hosts, where this donor DNA can undergo homologous recombination with the recipient genome, causing a transfer of any genetic markers encoded. Phage DNA is shown in blue, donor DNA in red, and recipient DNA in green.

with 3–4 headfuls produced on average from one concatemer. The phage tails are then attached to the filled proheads to complete the virion and the host bacterium lysed. The released phage, on adsorbing to a susceptible host, injects its DNA into the cell. The DNA undergoes circular permutation by recombination of the terminally redundant ends, to protect it from nucleases. Next, the DNA will either insert into the bacterial chromosome or remain as a plasmid as in the case of P1 if entering the lysogenic cycle or initiate the construction of new phage if entering the lytic cycle.

DNA Packaging in Transducing Particles

The random mispackaging of bacterial DNA that occurs in a generalized transducing phage happens infrequently. Estimates of the number of transducing particles in a P22 lysate from density transfer experiments show that they account for about 2% of all the particles, so that approximately 1 out of every 50 phage produced is a generalized transducing particle. Generalized transducing particles are thought to arise by one of two possible ways. First, the phage packaging apparatus recognizes *pac*-like sequences on the bacterial DNA, which are sufficiently similar to the phage *pac* site, and packages host, instead of phage, DNA. This is believed to be the stimulus for transducing particle formation in P22. However, there is little evidence to support this hypothesis for P1, and it seems most likely that, for this phage, the bacterial DNA is mispackaged from nicks or ends in the host DNA. Whichever the method of host DNA recognition, generalized transducing particles are filled in the same manner: by the headful, cleaving the DNA, and filling the next head from lengths of the bacterial chromosome or plasmid DNA. Again, the amount of host DNA that can be packaged and therefore transduced is dependent on the size of the phage head. P22 can transduce approximately 44 kb (around 1% of the host genome) in one transducing particle, whereas P1 is larger and can transduce around 115 kb (around 2% of the host genome), and the *B. subtilis* phage PBS1 is capable of packaging up to 300 kb (around 8% of its host genome). Any region of the host DNA can be packaged into a virion in this way although the frequency of transduction of different regions of the genome can vary. This is particularly noticeable if generalized transduction occurs through mispackaging from *pac*-like sites as the frequency of transduction of a particular marker depends on its location in the bacterial genome relative to a *pac*-like site. High-transducing (HT) mutants of P22 display an increased efficiency of transduction and this appears to be due to a reduced specificity in *pac*-site recognition and packaging. The resulting P22 HT particles transduce different regions of the chromosome with a similar frequency. Indeed, the mutations in P22 HT phage map to gene 3, which encodes the terminase required for *pac*-site recognition and initiation of subsequent DNA encapsidation. Therefore, mutations in packaging specificity determinants can improve and enhance transducing phage and support the model for P22 packaging DNA at sites related to *pac*. The only difference between a phage and a transducing particle is the origin of the DNA in its head so that once the host DNA is packaged, the remainder of the lytic cycle occurs as for the phage, up to the point of DNA injection into a susceptible host.

Fate of Transduced DNA

The fate of the transduced bacterial DNA differs from that of phage DNA once it has been injected into a new bacterium. These possible fates are listed below and summarized in **Figure 5**.

Abortive transduction

Following DNA injection, the majority of the DNA (90%) remains extrachromosomal within the recipient bacterium. In this case, the linear DNA is injected into the cell, along with a phage-encoded protein found in the prohead. The protein binds to the ends of the transduced DNA and circularizes it, protecting the DNA ends from host nucleases and preventing recombination with the recipient bacterial chromosome. The DNA can remain stable in this way for several generations and can even transcribe genes that are present on the DNA. However, abortively transduced DNA is unable to replicate, and is therefore inherited by only one daughter cell following division. So, any phenotype encoded in this region will be apparent in only a very small minority of the offspring, giving rise to minute colonies.

Abortive transductant DNA is only rarely able to recombine with the recipient chromosome. However, recombination may be stimulated if the DNA has been damaged. Nicks that have formed in the circularized DNA can promote the action of the host DNA repair system, resulting in recombination. For example, it has been shown that UV irradiation of generalized transducing phage lysates achieves a higher rate of stable transductants, due to the rendering of abortive transductants as recombinogenic.

Recycling of nucleotides

A small percentage of the DNA injected by transducing particles is left unprotected by the phage prohead proteins found in abortive transduction, and if this DNA is unable to undergo homologous recombination with the recipient bacterium, it is recycled by host degradation into component nucleotides that are incorporated into the bacterial genome during DNA repair. It has been estimated that not more than 15% of the transduced

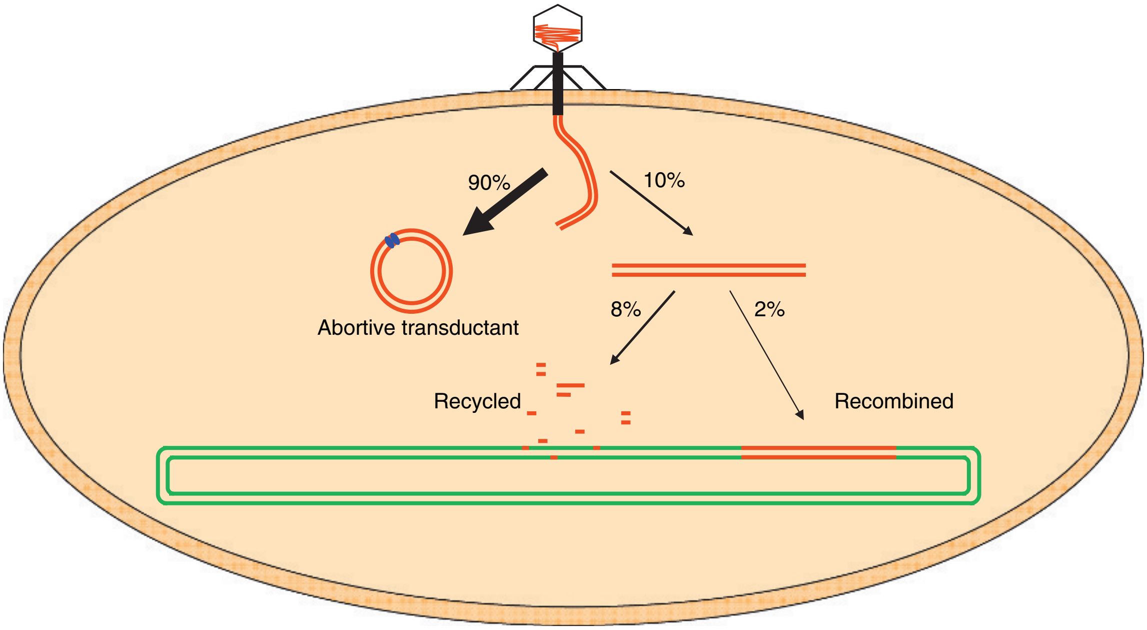

Figure 5 Fate of transferred DNA in generalized transduction. Once the donor DNA is injected into the cell from a transducing particle, there are alternative fates that it can undergo. The majority of the injected DNA will be protected by phage proteins, which bind to the ends of the transduced DNA and circularize it, protecting it from nucleases and recombination. This process, known as abortive transduction, accounts for the fate of 90% of the transduced DNA. Two percent of the unprotected DNA is able to undergo homologous recombination into the recipient genome in stretches of at least 500 bp, with the remaining 8% degraded to its constituent nucleotides and incorporated into the recipient DNA. Phage proteins are shown in blue, donor DNA in red, and recipient DNA in green.

DNA is liable to this degradation, and that usually only around 8% of total transduced DNA undergoes this process.

Chromosomal recombination

If the recipient bacterium is sufficiently related to the donor bacterium at the DNA level, stable insertion of the transduced DNA into the recipient chromosome can occur via homologous recombination. This process usually occurs within 1 hour of transducing particle infection in *E. coli* and *Salmonella*. After this time, successful recombination is unlikely to happen and any remaining transduced DNA is usually degraded and recycled. Chromosomal recombination and subsequent stable transductant formation requires RecA-dependent replacement of the equivalent DNA on the recipient chromosome by the donor DNA, leading to the expression of the corresponding encoded phenotype carried by the transduced DNA. Stable recombination into the chromosome is only a rare event; it has been shown for P1 and P22 that only about 2% of the bacterial DNA injected by a transducing particle normally undergoes homologous recombination into the recipient genome in continuous stretches of at least 500 bp.

Plasmid inheritance

Some, but not all, generalized transducing phage are able to transduce plasmids from one host to another. In this case, the plasmid DNA is injected by the transducing particle and recircularizes into a stable plasmid that replicates along with the new host and is inherited by daughter cells. Broad host range phage, which may not be able to successfully transduce chromosomal DNA into bacteria lacking sufficient genetic identity, may still be able to transduce plasmids between genetically diverse bacteria, as recombination and therefore genetic identity with the recipient bacterium, is not required.

Development of Other Generalized Transduction Systems

Virulent phage that use the headful packaging method are obvious targets for development into generalized transducing phage, and some such phage have been manipulated to make them generalized transducers. For example, the well-characterized *E. coli* phage T4, which packages its DNA by the headful mechanism but is not capable of transduction in its wild-type state, has been modified for use as a generalized transducing phage. Mutation of T4 genes encoding endonucleases prevents the degradation of host DNA upon T4 infection, thus enabling transduction. This simply means that the bacterial DNA is left sufficiently intact to allow mispackaging to occur, and as T4 does not require *pac* sites in order to package its DNA, it is able to package both bacterial and phage DNA equally well, making it an extremely efficient generalized transducer.

The model temperate phage λ, described earlier in this article as a specialized transducing phage, can also, with adaptation, make generalized transducing particles. Lambda does not use the headful packaging mechanism of most known transducers, but utilizes an alternative mechanism whereby the DNA concatemers are cleaved at a specific *cos* site found at either end of the phage genome. Whereas only one *pac* site is needed to initiate headful packaging and DNA cleavage, two *cos* sites, one at either end of the DNA, are required for λ packaging. Packaging is initiated by recognition of the first *cos* site. The second *cos* site signals the end of the DNA to be packaged and that the DNA should be cleaved. As with *pac*, mistakes in DNA packaging can occur if DNA sequences resembling *cos* sites are found on the bacterial chromosome. However, the chances of two *cos* sites being found on the host DNA, exactly the required length apart, are minimal. Therefore, in the absence of a ‘signal’ sequence to cleave the DNA, when λ does package bacterial DNA into its proheads by mistake, there is usually a protrusion of a length of DNA and the phage tails are unable to bind to make a functional transducing particle. Simple *in vitro* DNase treatment of the lysate containing such partially formed particles cleaves the excess DNA and the phage tails are then able to attach to the proheads to complete the generalized transducing particle. However, even with other manipulations, λ is only a poor generalized transducer and its preferred use as a laboratory tool is that of a specialized transducer.

Another highly studied phage, Mu, can also operate as a generalized transducing phage although it is perhaps better known for its qualities as a transposable element. This phage packages its DNA via a headful mechanism, and a *pac* site is required to initiate the packaging of its DNA into the prohead. The genome of phage Mu when it is packaged is found flanked by host DNA of variable sequence; a short region of up to 150 bp at the left-hand end and a larger region of up to 3 kb on the right-hand end. Mu-transducing particles are believed to arise primarily due to the mispackaging of host DNA as for other headful packagers. However, it cannot be ruled out that at least some transduction events observed for Mu are derived from recombination with host DNA present at the right-hand end. Generalized transduction is also possible with mini-Mu, a Mu derivative with the central region of the genome deleted, leaving only the ends intact plus the transposase *A* gene. In the presence of a helper Mu, mini-Mu can be induced and will be packaged into a prohead together with the adjacent bacterial chromosome to a total length of 39 kb of DNA. Ninety percent of transductants that arise following infection of a susceptible host with this mini-Mu/donor DNA transducing particle, result from RecA-dependent homologous recombination with the recipient DNA. The remaining 10% of transductants result from mini-Muduction, whereby the donor DNA has a copy of mini-Mu attached at each end, which can insert anywhere into the recipient DNA in the same way as for random transposon insertion. Homology between the donor and recipient DNA is not required for mini-Muduction, therefore allowing transduction of DNA between any species of bacteria that Mu is able to infect. Mu has a relatively broad host range, and is able to infect and replicate in many different bacteria, making it a very useful genetic tool, particularly for bacterial strains without an existing identified generalized transducing phage. Clearly, Mu can be considered to display properties of both generalized and specialized transducing phage, in addition to transposable elements.

A combination of experimental and bioinformatic approaches may be utilized in the search for generalized transducers for a particular bacterial strain. When trying to isolate generalized transducers, headful packaging phage can be enriched by using their reduced sensitivity to chelating agents. Indeed, sodium pyrophosphate has been successfully used in this way to isolate transducing phage for *Streptomyces venezuelae*. With advances in the number of phage genomes sequenced, searches for generalized transducers for certain bacteria could also be undertaken at the genome level. Terminases of many headful packagers can be recognized and classified into functional groups using comparative genomics, which may narrow the search for generalized transducers. As previously mentioned, the terminase gene product is involved in recognizing sequences in phage DNA and initiating the series of packaging events that result in mature phage particles. Therefore, generalized transducing phage terminases that have reduced specificity are better transducers, a prediction borne out with P22 HT gene 3 terminase mutant phage compared with parental P22. It may be worthwhile in the future to develop rational and randomized mutagenesis approaches on sequenced phage with predicted headful packaging strategies, with the aim of developing generalized transducers, by mutation of terminases (e.g., P22) or genes involved in host DNA degradation (e.g., T4).

For phage that are poor transducers, or when using markers that are only transduced at low frequencies, methods have been devised to increase the frequency of transduction. As already mentioned, the frequency of generalized transduction can be greatly enhanced by UV irradiation of the donor lysate, whereby damage is introduced into the transduced DNA to stimulate recombination with the recipient’s chromosome. Also, insertion of a phage *pac* site into the bacterial DNA will greatly increase the efficiency of transduction of that region. This is particularly useful when transducing plasmids, as these are often transduced at lower frequencies than chromosomal markers.

Variations on Transduction

Gene Transfer Agents

GTAs are a class of prophage-like elements that package short random segments of bacterial DNA. Following release from the donor cell, GTAs infect neighbors, thereby enabling the transduction of bacterial genes that are incorporated via homologous recombination. These agents are reported to promote 'constitutive transduction' or 'capsduction' and this generalized transduction phenomenon was first discovered in 1974 by Marrs while studying genetic recombination in *Rhodobacter capsulatus*. Since the original observation, GTAs have also been found in diverse bacteria, including *Brachyspira hyodysenteriae, Methanococcus voltae, Desulfovibrio desulfuricans*, and *Bartonella* spp.

A common feature of sequenced GTAs is that the genes encoding the phage particles are present in the bacterial genome; they encode all of the products required for assembly of the phage (structural genes), but lack early genes responsible for the replication of phage DNA. Upon assembly, these unusual phage-like particles typically package between 4.5 and 14 kb of random host DNA, depending on the particular GTA. However, the prophage-like gene clusters encoding the *R. capsulatus* and the *B. hyodysenteriae* GTAs are 14.1 and 16.3 kb, respectively, which is considerably larger than the DNA these particles can package (4.5 and 7.5 kb, respectively). In contrast, tailed dsDNA phage typically package at least 40 kb of DNA. Most of the GTAs characterized also have a small head morphology compared to tailed phage, ranging from 30 to 80 nm in diameter. Therefore, due to the reduced capsid size, even when these GTAs randomly package their own DNA at a frequency as low as any other chromosomal genes it is clear that they are unable to package their entire genome. The inability to package their entire genome and the lack of early replication genes result in phage-like particles that are nonreplicative and, hence, do not form plaques on any host tested. As such, the genes encoding these phage-like elements are inherited in a predominantly vertical fashion from parent to daughter cells. Rare cases of GTA horizontal transfer have been inferred in phylogenetic studies using the *R. capsulatus* GTA, but the mechanisms are unknown. Once the GTA transducing particles are formed, it is unclear how they are released from donor cells because detection of these particles does not usually correlate with lysis of the host bacterium. Indeed, the sequence of the *R. capsulatus* GTA is not predicted to encode homologues of phage lysin or holin genes. Conversely, the *B. hyodysenteriae* GTA possesses copies of lysin and holin genes and purified lysin was shown to disrupt cell walls by degrading peptidoglycan.

The theory that these 'defective' prophage-like elements are actually host-adapted gene transfer modules is supported by regulation studies. Work on *R. capsulatus* has demonstrated that its GTA is controlled by the CckA/CtrA two-component phosphorelay regulatory system that also controls motility. In addition, expression of the GTA structural genes is activated by a LuxIR-type quorum sensing system that enhances gene transfer in a cell-density-dependent manner in response to the *N*-hexadecanoyl-homoserine lactone signal. Therefore, gene transfer is promoted when there is a large number of signal-related recipients nearby, which would increase the chance of a successful DNA transfer event. Finally, the presence of *R. capsulatus*-like GTA sequences in many α-proteobacteria indicates functional selection for their maintenance in diverse genomes.

The limited functional studies and sequence data for most GTA elements highlight a paucity of information on this unusual mode of 'generalized transduction'. Further studies on GTAs could provide more information on DNA packaging mechanisms and transduction. Indeed, phylogenetic studies have demonstrated that homologues of the *R. capsulatus* GTA terminase product cluster in a single group, which presumably represent enzymes with reduced sequence specificity proficient in packaging random host DNA. If phage that contain GTA-like terminases are identified, it might indicate the potential of these phage to function in generalized transduction. Areas requiring further analysis include the mechanisms of relaxed packaging specificity, particle release, and adsorption to bacteria. It is likely that many more diverse GTAs exist in other bacteria, which await discovery through both bioinformatics and functional studies.

Are 'Cargo' Genes a Special Case of Transduction?

The recent genome sequencing efforts of both bacteria (including their prophage) and phage have revealed that many phage and prophage genomes contain nonessential genes of putative bacterial origin. Prophage-associated genes often impart an advantage to bacterial lysogens via the expression of toxins or virulence factors that aid bacterial pathogenesis. This phenomenon is called lysogenic conversion due to the 'conversion' of the bacterial host upon lysogeny. A familiar example is the prophage-encoded shiga toxin produced by certain pathogenic *E. coli* lysogens. Alternatively, phage-encoded gene products, of bacterial origin, can aid in phage lytic infection. One example is the production of photosynthetic proteins by certain cyanophage upon infection of cyanobacteria. The phage-encoded photosystem genes (e.g., *psbA* and *psbD*) are thought to increase energy production during phage infection of the cyanobacterial host, ultimately assisting in phage replication. Phylogenetic and host range studies demonstrate that these photosystem genes were acquired by phage from bacteria, have been shuffled among the phage (probably via coinfection), and in some cases have been recombined back into some cyanobacteria. The generation of a bacterial recombinant following infection with a virulent phage must only arise when the infection is nonproductive. Therefore, it is theoretically possible to define both virulent and temperate phage that carry these 'cargo' genes (or 'morons') as a form of potential transducing phage, since these genes may be acquired and maintained in the infected bacteria. Obviously, this sort of genetic promiscuity or modularity in phage genomes represents the dynamic evolution of these biological entities and it is interesting to consider how these 'cargo' genes might have been acquired (e.g., by illegitimate recombination and/or incorrect prophage excision). Clearly, phage are a constant source of genetic mixing that often blurs the boundaries between what are considered phage and bacterial genes. When reassessed in this broader context of general phage evolution, many more phage might be considered as transducers, albeit at a very low frequency compared to the well-characterized classes of specialized and generalized transducing phage.

Transduction as a Genetic Tool

Since the discovery of phage, researchers have rapidly harnessed their knowledge of phage biology for the development of new tools and applications. Examples include phage (and their products) as antibacterial agents, as DNA delivery vehicles for expression of particular genes in a desired host (e.g., for transposon delivery or luciferase reporter expression), and as components of DNA cloning, integration, and expression systems. These cases represent just a handful of phage uses, with those related to their transducing (especially generalized) properties being covered here.

Isolation and Characterization of Generalized Transducing Phage

Obviously, the first requirement to enable the use of a generalized transducing phage is to isolate one for the bacterium of interest. There is now extensive evidence of the ubiquitous distribution of phage in the natural environment. In fact, it is estimated that there are approximately 10 phage for each bacterium on Earth. Therefore, a good starting point for phage isolation is the native environment from which the bacterium was originally cultured. For enteric bacteria, a good source of phage is raw and treated sewage. Using a variety of sources, with the target bacteria as an indicator, phage have been isolated for many bacterial genera. Phage are then screened for transducing ability using, for example, donors with transposon insertions in defined genes with screenable phenotypes (e.g., auxotrophy). Transduction is performed in a wild-type recipient, selecting for the transposon antibiotic resistance marker. Putative transductants are then screened for cotransduction of the phenotype (e.g., auxotrophy on minimal media). In addition, transduction of plasmids can be tested. Transduction of multiple loci is required to confirm isolation of a generalized transducing phage.

Using strategies similar to those described above, generalized transducing phage have been isolated for strains of many bacterial genera, including *Bacillus, Caulobacter, Citrobacter, Erwinia, Myxococcus, Pseudomonas, Salmonella, Serratia, Staphylococcus, Streptococcus,* and *Streptomyces*. In these cases, and others, generalized transducing phage have enhanced the genetic tractability of their host organisms. Below, the most common uses of generalized transducing phage are discussed.

Constructing Strains

The ability to transduce loci with selectable or screenable phenotypes has greatly facilitated bacterial genetic manipulations and the power of generalized transduction has been aided particularly by its use in conjunction with transposon mutagenesis. Typically, following transposon mutagenesis, it is desirable to confirm the presence of a single transposon insertion, which can be checked by Southern blot analysis. However, it is also possible that other secondary (e.g., point) mutations may have arisen in the mutant strain. In order to move the transposon insertion of interest into a background likely to be free from either secondary transposons or other mutations, generalized transduction is the facile method of choice. Cotransduction of the marker and the phenotype studied confirms that the appropriate transposon copy has been selected. Furthermore, where selectable markers are available, generalized transduction makes the construction of double and multiple mutant strains quick and easy compared with alternative marker (allelic) exchange procedures. Currently, in most molecular microbiology laboratories in a postgenomics era, this is the major use of generalized transducing phage.

Localized Mutagenesis

To examine gene function in detail, it is necessary to analyze the effects of mutations. There is often value in studying more subtle mutations than those generated by knockout or transposon disruption. In the current era of molecular biology, localized mutagenesis can be performed by error-prone polymerase chain reaction (PCR) followed by a strategy to recombine mutated sequences in a single copy into the bacterial chromosome. Although this is a powerful and 'clean' technique, it relies on suitable delivery (e.g., transformation and/or conjugation) systems for the organism studied. An alternative strategy is to subject the bacterium to UV or chemical mutagenesis and screen for mutants. However, any interesting mutants may contain other mutations elsewhere in the genome, complicating further analysis. If the gene of interest is located in the vicinity of a selectable marker (e.g., a transposon), it can be transduced from the mutated strain into an unmutagenized background, selecting for the marker and, by linkage, any nearby mutations in the gene of interest. Alternatively, a transducing lysate, prepared on the transposon-tagged strain, can itself be exposed to mutagenic agents such as hydroxylamine or nitrous acid. Therefore, only the DNA packaged within the phage is mutated and upon transduction into a recipient will be linked to the selectable marker. Due to the relatively short stretches of DNA transferred by transduction, mutations linked to the selected marker can be identified and characterized.

Genetic Mapping

Transductional mapping was used in *E. coli* and *Salmonella* to determine the fine genetic structure of closely linked genes and mutations within genes. With the huge expansion and ease of genome sequencing, there is now little need for these traditional genetic mapping experiments. The basic principle of transductional mapping relies on measuring the genetic linkage of loci that depends on the efficiency of cotransduction and recombination into particular recipients. Simply, if two mutations are linked (within the size of DNA packaged in the phage capsid), they will be transduced together at a particular frequency. Calculation of linkage depends on the distance of the loci from each other and the number of recombination events required to isolate a transductant. Although historically interesting and extremely powerful in early bacterial genetics, these techniques are no longer widely pursued as their utility has to a large extent been overtaken by advances in molecular biology methods.

Plasmid Transduction

For many genetic manipulations in bacteria, it is necessary to introduce plasmids into recipient cells. Commonly, conjugation and transformation are the systems of choice for plasmid transfer. However, phage-mediated plasmid transduction can be a useful method for bacterial strains with poor transformation efficiency or a paucity of suitable conjugal plasmids. Transduction of plasmids has been demonstrated with many generalized transducing phage. However, the efficiency can vary greatly depending on the phage and the size and sequence of the transduced plasmid. The currently accepted model is that multimeric double-stranded plasmid DNA, generated during rolling circle replication, is accidentally packaged by the phage. After injection of the plasmid DNA, the recipient bacterium regenerates the plasmid in a process requiring homologous recombination and RecA.

For efficient transduction of pBR322 by wild-type P22, it is necessary to introduce a *pac* sequence. Furthermore, a P22 HT derivative, with reduced packaging specificity, can transduce pBR322 without any cloned DNA fragments. The plasmid transducing mechanism has also been analyzed for a modified T4 that is proficient in generalized transduction (see 'Generalized transduction'). Transduction of pBR322 by this modified T4 involves packaging of the equivalent of 38 monomers of the plasmid arranged as multimers and establishment in the recipient requires homologous recombination. Phage P1 can package plasmids that contain a P1 *inc* site and, due to the broad host range of P1 (see below), transduce these into a variety of strains.

In the absence of *pac* sequences, pBR322 requires cloned P22 DNA fragments for efficient plasmid transfer by P22. The method of transduction involves an initial homologous recombination step between P22 and the plasmid in the donor prior to transduction into the recipient. This dramatic increase in transduction efficiency, when a portion of phage DNA is cloned into plasmids, has also been observed for multiple bacteria, including species of *Bacillus, Lactobacillus, Staphylococcus*, and *Streptomyces*.

Intergeneric Gene Transfer

Phage–host receptor interactions are often highly specific with a phage only recognizing a single strain of a given species. In other cases, phage can have a very broad host range, with P1 and Mu providing classic examples. The obvious benefit of isolating a broad host range transducing phage is that it is possible to use it for multiple bacterial isolates. Phage P1 has an invertible region that can switch the expression between two alternative tail fiber products, a major determinant in phage–host interactions. Depending on what tail fiber form is expressed, P1 can adsorb to, and replicate in, *Citrobacter, Enterobacter, E. coli, Erwinia, Klebsiella, Pseudomonas*, and *Salmonella*. P1 can also inject its DNA into strains of *Agrobacterium, Flavobacterium, Myxococcus*, and *Vibrio* but cannot produce phage progeny on these. In this manner, plasmids have been introduced into *Myxococcus* by P1, where they are unable to replicate. This is the basis of a suicide vector delivery system that is used for transposon mutagenesis and targeted gene disruptions in these bacteria.

Intergeneric gene transfers between *E. coli* and *Salmonella* have been performed using P1. Some researchers have used the transduction efficiency as a rough indicator of DNA homology between donor and recipient. However, these transductions are often unsuccessful due to the large genomic differences between these genera and the requirement of long stretches of near-identical sequence for efficient homologous recombination. Analysis of the bacterial transductants shows that recombination has frequently occurred between highly conserved regions, such as the ribosomal (*rrn*) loci, which can result in large genome alterations. It is now understood that the recipient's methyl-directed mismatch repair system is responsible for some of the recombinational stringency and, as such, mismatch repair mutants are more efficient recipients of donor DNA from different genera. To date, such intergeneric gene transfer experiments have not been widely utilized outside of *E. coli* and *Salmonella.*

Lambda as a DNA Delivery Vehicle

Lambda has been developed as an efficient transduction tool for the introduction of DNA into host cells. This includes the packaging and transduction of cosmids and transposons to target cells. Cosmids are plasmid vectors that contain λ *cos* sites, hence their hybrid name. Genomic libraries can be generated by ligation of large chromosomal DNA fragments (up to 47 kb) into cosmids. Packaging of the cosmids is performed *in vitro* with λ capsids via the recognition and cleavage at consecutive *cos* sites. Phage tails are attached and the completed particles can then transduce the cosmid into a suitable recipient, where it is replicated by virtue of a plasmid origin of replication. These cosmids can be further packaged *in vivo* following infection with λ. In a similar mechanism, λ derivatives containing transposons (e.g., Tn5, Tn10, Tn*phoA*, and Tn*blaM*) can be transduced into a target bacterium. Transposition (mutagenesis) events can be selected and mutants subsequently characterized. Lambda replication is inhibited by amber mutations in the phage morphogenesis genes, whereas in some other genera (see below) wild-type λ is unable to replicate. Compared with plasmid-based (conjugation) mutagenesis procedures, λ-based systems are faster and have no requirement for counterselection of *E. coli* donors.

The requirement of recipient bacteria for the LamB phage λ receptor originally restricted the host range use of λ. However, a wider range of Gram-negative hosts for λ adsorption and DNA injection have been generated by introducing the *lamB* gene on a suitable plasmid. Bacteria capable of expressing and transporting LamB to the outer membrane may then be infected by phage λ. Plasmids containing *lamB* have enabled λ infection of species of various genera, including *Agrobacterium, Erwinia, Klebsiella, Mesorhizobium, Pseudomonas, Salmonella, Serratia*, and *Vibrio*, making delivery of cosmids and transposons relatively straightforward in these systems. In an interesting extension of the λ host range, virons can be taken up by certain eukaryotic cells in culture and also by antigen presenting cells *in vivo.* This mechanism enables the delivery of cosmids and other vectors into eukaryotic cells for expression studies and vaccine delivery.

Transduction in the Environment

With increasing numbers of sequenced bacterial genomes and advances in comparative genomics, it is now clear that HGT accounts for a large degree of the genetic diversity found within bacterial species. Phage are believed to be the most abundant biological entities, with estimates of 10^{31} phage on the planet. The number of transduction events per year has been estimated to be 10^{14} in the Tampa Bay Estuary and 10^{13} in the Mediterranean Sea. This demonstrates just how important the role of phage-mediated transduction might be for HGT in the natural environment. Indeed, interrogation of the genomes of nearly all sequenced bacteria reveals the presence of numerous prophage and prophage-like elements, and these regions are often associated with adjacent horizontally acquired regions and 'cargo' genes of bacterial origin. Possible explanations for these 'cargo' genes is that specialized transduction or illegitimate recombination has taken place. Some of these phage-transferred regions encode bacterial virulence factors that can convert the host into a pathogenic strain. For example, it is known that the cholera toxin is encoded on a phage, CTXΦ, and infection of a nonpathogenic *Vibrio cholerae* strain with this phage will render it pathogenic. More recently, generalized transducing phage have been shown to transduce both the VPI pathogenicity island, which encodes the receptor for CTXΦ, allowing adsorption and infection of strains that are normally resistant to this phage, and the genes encoding CTXΦ itself.

Unfortunately, since transduction frequently relies on homologous recombination between highly related sequences, in these instances, its impact on bacterial genomes is not readily detected using current bioinformatic sequence tools. Therefore, the importance of transduction in bacterial evolution has been inferred from a combination of laboratory experiments, an understanding of the global abundance of phage, and the detection of transduction in the natural environment.

Several studies have shown transduction of both chromosomal and plasmid DNA to occur in a variety of natural environments. Transduction of both plasmid and chromosomal markers between strains of *Pseudomonas aeruginosa* has been demonstrated in freshwater environments, as well as between bacteria on leaf surfaces, even when the donor and recipient were originally on different plants. Broad host range phage have been shown to transduce plasmids among a diverse range of bacteria in natural populations of both fresh and marine water environments. It has also been reported that chromosomal markers have been transferred from virus-like particles, spontaneously released from five strains of marine bacteria, to convert different auxotrophic mutants of *E. coli* to prototrophy. This suggests generalized transduction between bacteria of different families, although these conclusions were not reinforced by verification of the corresponding prototrophic gene acquisition. Similar studies on virus-like particles isolated from thermal vents and hot spring bacteria have also been undertaken, and the same phenomenon observed. If verified, these findings would indicate that generalized transduction can occur between a broad range of bacterial species in the environment. Generalized transducing phage have also been detected containing various bacterial 16S rRNA genes, which are bacterial species-signature-specific sequences. This could imply a role for generalized transduction in the horizontal transfer of 16S rRNA gene sequences between bacteria of different genera.

Many phage are very stable and resistant to degradation in the environment, particularly in comparison to naked DNA, and transducing particles thereby represent a comparatively stable repository for bacteria DNA. Given these observations, together with the global abundance of phage, it seems possible that phage-mediated transduction events in the environment are a major force driving HGT and adaptive evolution of bacteria.

Conclusion

The phage-mediated transfer of bacterial DNA between donor and recipient cells is a form of HGT called transduction. The current understanding of the molecular mechanisms of transduction has been fueled by studies of a small number of 'model' phage–host systems. With advances in modern molecular biological techniques (many of which are themselves derived from the products of phage research), it is important not to forget the power of simple genetic techniques that exploit phage. Indeed, development of a generalized transducer is still an extremely useful tool for genetic analysis of any bacterial strain. A recent stimulation in phage research and the '-omics era' has provided major advances in knowledge of the genomes, evolution, ecology, and diversity of phage. For example, the host-adapted function of GTAs is a salient reminder that some sequences that may appear like 'defective' prophage in bacterial genomes can in fact be highly effective HGT mechanisms proficient in generalized transduction. It is anticipated that the recent rejuvenation in phage biology research will continue to further our appreciation and understanding of transduction, both as a tool for bacterial geneticists and as an important evolutionary force in the adaptation of phage and their bacterial hosts.

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Transport, Solute

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Introduction
Transporter Classification and Annotation
Major Solute Transporter Families
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Soil/Plant-Associated Microbes
Autotrophs
Conclusion
Further Reading

Abbreviations

ABC	ATP-binding cassette
EI	enzyme I
EII	enzyme II
GlpT	glycerol-3-P:P antiporter
HPr	phosphoryl carrier protein
LacY	lactose:H^+ symporter
MFS	major facilitator superfamily
NBD	nucleotide-binding domain
ORF	open reading frame
PEP	phosphoenolpyruvate
PTS	phosphotransferase system
SAM	*S*-adenosylmethionine
TC	transporter classification
TCA	tricarboxylic acid
TDG	β-D-galactopyranosyl-1-thio-β-D-galactopyranoside
TM	transmembrane
TMD	transmembrane domain
TMS	transmembrane segment

Introduction

The cellular membrane is a selectively permeable barrier between the cell and the extracellular environment. Cytoplasmic membrane transporters are a group of membrane proteins embedded in the phospholipid bilayer through multiple α-helical segments of 20–25 hydrophobic amino acids. They mediate the movement of molecules and ions across cytoplasmic membrane. These transporters play essential roles in fundamental cellular processes like the acquisition of organic nutrients, extrusion of toxic and waste compounds, maintenance of ion homeostasis, environmental sensing, and cell communication in archaea, bacteria, and eukaryotes. Typically, 3–16% of open reading frames (ORFs) in prokaryotic genomes are predicted to encode membrane transport proteins, emphasizing the importance of membrane transport to cellular lifestyles. Thus, knowledge of the suite of membrane transporters present in the organism is important to fully understand an organism's metabolism and physiology as well as its adaptations to its natural ecological niches.

Various transport systems differ in their putative membrane topology, energy-coupling mechanism, and substrate specificities. Membrane channels form open pores through the membrane and mediate a facilitated diffusion of water, specific types of ions, or hydrophilic small molecules down their concentration or electrical gradient. This process is not coupled to metabolic energy and cannot transport against the concentration gradient across the membrane. The general characteristics of channels include rapid rates of transport, high selectivity as the narrow pores in the channel restrict passage to ions of the appropriate size and charge; and most of the ion channels are not permanently open, a property referred to as gated channels. They open transiently in response to specific stimuli, for example, the binding of neurotransmitters or other signaling molecules (ligand-gated channels), and the changes in electric potential across the plasma membrane (voltage-gated channels). Primary active transporters mediate transport via an energy-dependent active transport process. Transport involves the binding of specific molecules on one side of the membrane, then the transporter undergoes a conformational transformation that allows the substrate to pass through the membrane and release into the other side of the membrane. It exhibits a much lower rate of transport and higher substrate stereospecificity compared to the channels. The most common energy-coupling mechanism for primary active transporters is the utilization of ATP. The secondary transporters also mediate energy-dependent active transport process, but utilize a secondary source of energy (e.g., an ion/solute electrochemical gradient). Secondary transporters mediate the transport of their substrates through the processes like uniport, symport, or antiport. Uniporters transport a single molecule without the involvement of other molecules. Symporters transport two or more molecules at the same time and in the same direction. Antiporters mediate the exchange of one or more molecules for others. Additionally, there are group translocation systems which are transporters that modify their substrates during transport, exemplified by the bacterial phosphotransferase system (PTS) transporters. PTS transporters typically enable the uptake of sugars from the external environment and concomitant phosphorylation and release into the cytoplasm as sugar phosphates, using phosphoenolpyruvate (PEP) as both energy source phosphoryl donor and energy source.

A great variety of substrates can be transported across the membrane by transporters. These include inorganic molecules; carbon compounds; amino acids and derivatives; bases and derivatives; vitamins, cofactors, signaling molecules and their precursors; drugs, dyes, sterols, and toxic substances; and macromolecules. **Figure 1** shows the predicted metabolic and transport capabilities of the soil- and plant-associated bacterium *Pseudomonas putida* and provides an example of the transporter complexity found in microorganisms.

The structural study of membrane transporters has lagged behind compared to other types of proteins. Owing to their amphipathic characteristics, membrane transporters have proven difficult to crystallize, as required for three-dimensional structural analysis by X-ray diffraction. The percent of membrane transporter structures deposited into PDB is much less than the percent of transporters in the genome. Indeed it is only in recent years that structures of cytoplasmic membrane transporters have started to become more readily available. KcsA, a potassium ion selective bacterial ion channel, was among the first several membrane transporters with the high-resolution structure solved. Its structure was first reported in 1998 by MacKinnon's group utilizing X-ray crystallography, and later the refined structure was reported at 2.0 Å resolution. The KcsA channel is a homotetramer with a fourfold symmetry axis forming the potassium ion permeation pathway. The N- and C-termini of each subunit are located inside the bacterial membrane, contains two integral transmembrane helices, which are connected by a P-loop. The P-loop consists of a short helix that spans about 10 Å into the membrane and a loop region. It forms the selectivity filter, which is responsible for the selectivity for potassium ion over other ions. The structural studies of KcsA channel, as well as other potassium ion channels (MthK, KvAP, and KirBac), suggest the involvement of protein backbone structures in the selectivity process rather than individual amino acid residues, and illustrate the mechanism of high flux rate and selectivity of small polar and charged molecules across cell membranes.

High-throughput genomic and bioinformatics analyses provide important tools to facilitate the study of membrane transporters. In stead of focusing on one or several transporter genes at a time, this type of study emphasizes the big picture, that is, the complete transporter profile. Examples include systemic annotation and classification of transporters; comparative study of the fundamental differences of transporter features among organisms with different evolutionary background; the association of genome transporter features with evolutionary history, physiology, and lifestyle; and the integration of transporter reactions into metabolism network.

In this article, we will review the features and functions of major transporter types and families, with the focus on the recent progresses in structural and functional studies. We will also overview the bioinformatic classification of transporters as well as other types of comparative genomic studies of membrane transporters.

Transporter Classification and Annotation

Transporter Classification System

Transporters with similar functions characteristically cluster together in phylogenetic analyses. Thus, the substrate specificity appears to be a relatively conserved evolutionary trait in transporters. This has led to the premise that phylogeny can provide a rational basis for functional assignment. The transporter classification (TC) system represents a systematic approach to classify membrane transporter families according to the mode of transport, energy-coupling mechanism, molecular phylogeny, and substrate specificity. The TC system is analogous to the enzyme commission system for classification of enzymes, except that it incorporates both functional and phylogenetic information. Transport mode and energy-coupling mechanism serves as the primary base for the classification due to their relatively stable characteristics. There are four major characterized classes of solute transporters in the TC system: channels, secondary transporters, primary active transporters, and group translocators (**Figure 2**). Transporters of unknown mechanism or function are included as a distinct class.

- Class 1. Channels/pores: Channels are energy-independent transporters that transport water, specific types of ions, or hydrophilic small molecules down the concentration or electric gradient with higher rates of transport and lower stereospecificity

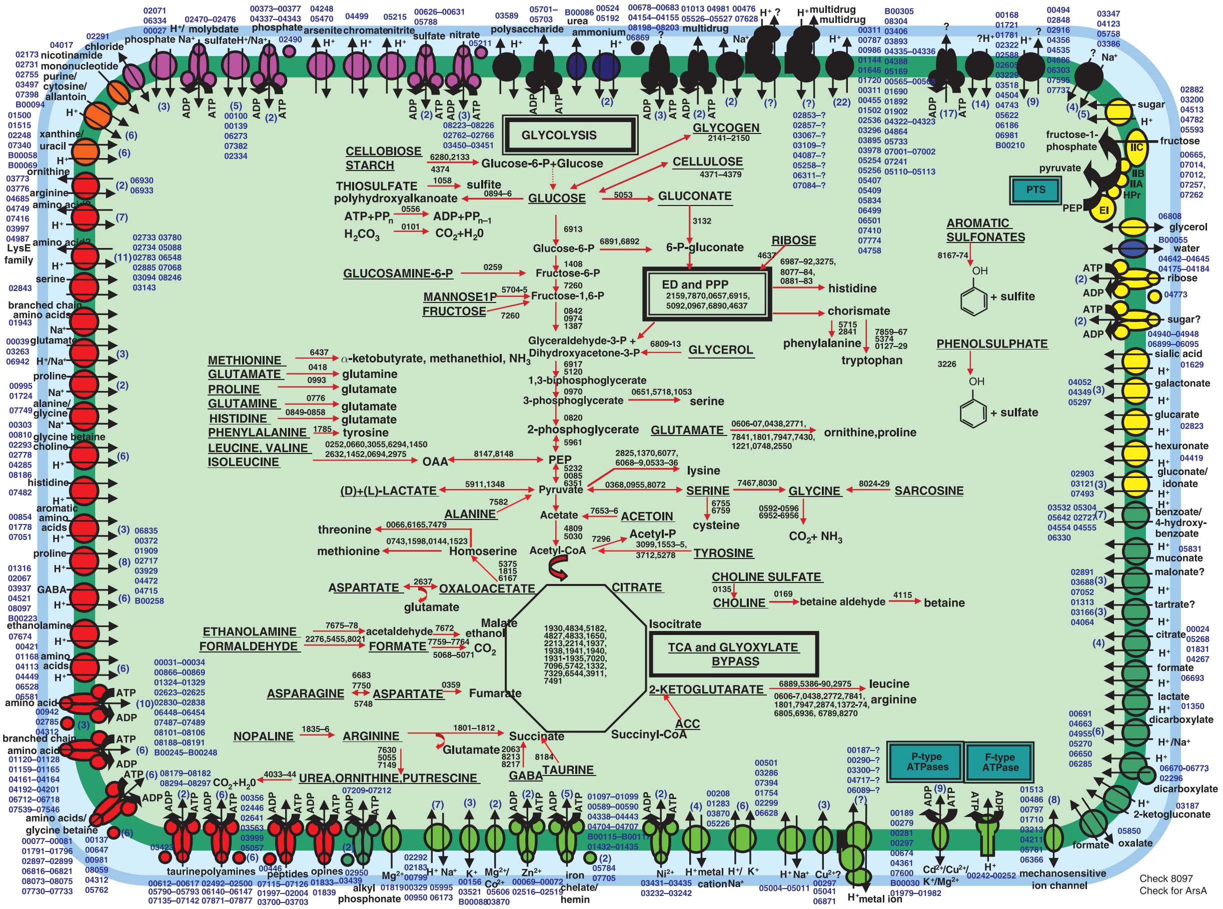
GLYCOLYSIS
CELLOBIOSE
STARCH
Glucose-6-P+Glucose
GLYCOGEN
CELLULOSE
THIOSULFATE
polyhydroxyalkanoate
sulfite
GLUCOSE
GLUCONATE
Glucose-6-P
6-P-gluconate
RIBOSE
GLUCOSAMINE-6-P
Fructose-6-P
MANNOSE1P
FRUCTOSE
Fructose-1,6-P
ED and PPP
histidine
chorismate
phenylalanine
tryptophan
AROMATIC SULFONATES
+ sulfite
PHENOLSULPHATE
+ sulfate
Glyceraldehyde-3-P + Dihydroxyacetone-3-P
GLYCEROL
METHIONINE
GLUTAMATE
PROLINE
GLUTAMINE
HISTIDINE
PHENYLALANINE
LEUCINE, VALINE
ISOLEUCINE
α-ketobutyrate, methanethiol, NH₃
glutamine
glutamate
tyrosine
OAA
1,3-biphosphoglycerate
3-phosphoglycerate
serine
2-phosphoglycerate
GLUTAMATE
ornithine,proline
PEP
lysine
Pyruvate
SERINE
GLYCINE
SARCOSINE
(D)+(L)-LACTATE
ALANINE
Acetate
ACETOIN
cysteine
CO₂+ NH₃
threonine
methionine
Homoserine
Acetyl-CoA
Acetyl-P
TYROSINE
ASPARTATE
OXALOACETATE
glutamate
CITRATE
CHOLINE SULFATE
CHOLINE
betaine aldehyde
betaine
Malate
Isocitrate
ETHANOLAMINE
FORMALDEHYDE
acetaldehyde
ethanol
FORMATE
CO₂
TCA and GLYOXYLATE BYPASS
ASPARAGINE
ASPARTATE
Fumarate
2-KETOGLUTARATE
leucine
arginine
NOPALINE
ARGININE
Succinate
Succinyl-CoA
ACC
Glutamate
GABA
TAURINE
UREA,ORNITHINE,PUTRESCINE
P-type ATPases
F-type ATPase
PTS
sugar
fructose
fructose-1-phosphate
pyruvate
glycerol
water
ribose
sugar?
sialic acid
galactonate
glucarate
hexuronate
gluconate/ idonate
benzoate/ 4-hydroxy-benzoate
muconate
malonate?
tartrate?
citrate
formate
lactate
dicarboxylate
dicarboxylate
2-ketogluconate
oxalate
formate
mechanosensitive ion channel
nicotinamide mononucleotide
purine/ cytosine/ allantoin
xanthine
uracil
ornithine
arginine
amino acids
LysE family
serine
branched chain amino acids
glutamate
proline
alanine/ glycine
glycine betaine
choline
histidine
aromatic amino acids
proline
GABA
ethanolamine
amino acids
amino acids
branched chain amino acids
amino acids/ glycine betaine
taurine
polyamines
peptides
opines
alkyl phosphonate
Mg²⁺
K⁺
Zn²⁺
iron
chelate/ hemin
Ni²⁺
metal cation
Cd²⁺/Cu²⁺/ K⁺/Mg²⁺
metal ion
chloride
molybdate
phosphate
sulfate
arsenite
chromate
nitrite
sulfate
nitrate
polysaccharide
urea
ammonium
multidrug
Check 8097
Check for ArsA

Figure 1 Metabolic reconstruction of pathways present in the *Pseudomonas putida* genome. Transporters are grouped by substrate specificity as follows: inorganic cations (green), inorganic anions (pink), carbohydrate/carboxylates (yellow), amino acids/peptides/ amines/purines/pyrimidines (red), and drug efflux and others (black). Question marks indicate uncertainty about the substrate transported. Export or import of solutes is designated by the direction of the arrow through the transporter. The energy-coupling mechanisms of the transporters are also shown: solutes transported by channel proteins are shown with a double-headed arrow; secondary transporters are shown with two-arrowed lines, indicating both the solute and the coupling ion; ATP-driven transporters are indicated by the ATP hydrolysis reaction; and transporters with an unknown energy-coupling mechanism are shown with only a single arrow. Components of transporter systems that function as multisubunit complexes, which were not identified, are outlined with dotted lines. Where multiple homologous transporters with similar substrate predictions exist, the number of that type of transporter is indicated in parentheses. Systematic gene numbers (XXXXX) are indicated next to each pathway or transporter; those separated by a dash represent a range of consecutive genes. The outer and inner membranes are sketched in dark blue and dark green respectively, the periplasmic space is indicated in light turquoise, and the cytosol in light green. ADP, adenosine diphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; FucNAc, *N*-acetylfucosamine; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GluNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NeurNAc, *N*-acetylneuraminate; P, phosphate; PP, diphosphate; Pyr, pyruvate. Reproduced with permission from Environmental Microbiology. Nelson KE, Weinel C, Paulsen IT, *et al*. (2003) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environmental Microbiology* 4(12): 799–808.

compared to other transporter classes. Most of the ion channels, referred to as gated channels, open only in response to specific chemical or electric signals.

- Class 2. Electrochemical potential-driven transporters (secondary transporters): Secondary transporters (also called carriers) utilize an ion or solute electrochemical gradient, for example, proton/sodium motive force, to drive the transport process. Uniporters move a single type of molecule down its concentration gradient. Antiporters and symporters couple the transport of ion or molecule against its concentration gradient with the movement of one or more different ions or molecules down its gradient.
- Class 3. Primary active transporters: Primary active transporters couple transport process to a primary source of energy, such as a chemical reaction (e.g., ATP hydrolysis), and move substrates across membrane against a chemical concentration gradient or electric potential or both.

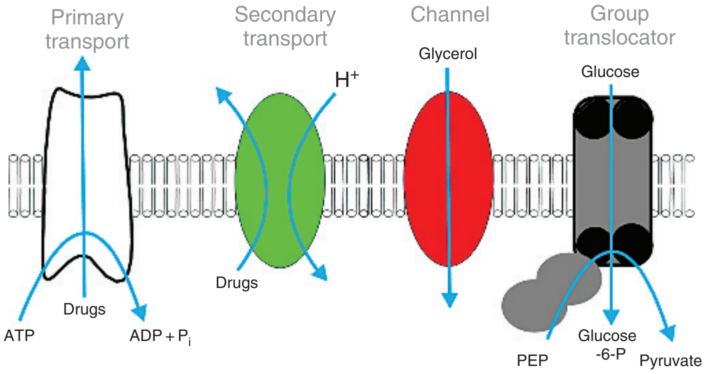

Figure 2 Representative examples of the four main types of transporters. The examples are as follows: primary transporter, *Lactococcus lactis* LmrA multidrug efflux pump; secondary transporter, *Staphylococcus aureus* QacA multidrug efflux transporter; channel, *Escherichia coli* GlpF glycerol channel; and group translocator, *E. coli* PtsG/Crr glucose PTS transporter.

- Class 4. Group translocators: Group translocators modify their substrates during the transport process. The bacterial PTS is the only characterized transporter family in this class. It phosphorylates the sugar substrates using PEP as the phosphoryl donor and the energy source, and releases them into cytoplasm as sugar phosphates.
- Class 5. Incompletely characterized transporter systems: Transporter protein families for which insufficient information is available to allow classification in a defined class belong to this class.

Each transporter class is further classified into individual families or superfamilies according to their function, phylogeny, and/or substrate specificity. Specific transport protein can be represented by a TC number, which normally has five components, V.W.X.Y.Z. V (a number) corresponds to the transporter type (i.e., channel, secondary transporters, primary active transporters, or group translocators); W (a letter) corresponds to the transporter subtype, which in the case of primary active transporters refers to the energy source used to drive transport; X (a number) corresponds to the transporter family; Y (a number) corresponds to the subfamily in which a transporter is found; and Z (a number) corresponds to the substrate or range of substrates transported. For example, the well-characterized *Escherichia coli* lactose:H$^+$ symporter (LacY) lactose permease is represented in the TC system as 2.A.1.5.1, where '2' indicates LacY is a secondary transporter, 'A' indicates it is a uniporter/symporter/antiporter, '1' indicates it belongs to the major facilitator superfamily (MFS), '5' indicates it belongs to an oligosaccharide symporter subfamily within the MFS family, and the last digit '1' indicates LacY is a lactose/proton symporter.

TCDB: A TC Database

Transport system families included in the current TC system are available in database format on the web. The TCDB site provides detailed information and references for each transporter class, subclass, family, subfamily, and individual proteins (**Table 1**). When possible, these

Table 1 The top 12 largest transporter families in the TransportDB database. Most of them are widely distributed in bacteria, archaea, and eukaryotic species, with the exception of SSPTS (bacteria) and MC (eukaryotes)

Family ID	*Family name*	*TC#*	*Count*	*Occurrence*[a]
ABC	The ATP-binding cassette superfamily	3.A.1	37 185	B, A, E
MFS	The major facilitator superfamily	2.A.1	8983	B, A, E
F-ATPase	The H$^+$- or Na$^+$-translocating F-type, V-type, and A-type ATPase superfamily	3.A.2	3153	B, A, E
DMT	The drug/metabolite transporter superfamily	2.A.7	2695	B, A, E
SSPTS	Sugar-specific phosphotransferase system	4.A	2388	B
P-ATPase	The P-type ATPase superfamily	3.A.3	1881	B, A, E
APC	The amino acid–polyamine–organocation family	2.A.3	1854	B, A, E
RND	The resistance-nodulation-cell division superfamily	2.A.6	1699	B, A, E
MOP	The multidrug/oligosaccharidyl-lipid/polysaccharide flippase superfamily	2.A.66	1460	B, A, E
TRAP-T	The tripartite ATP-independent periplasmic transporter family	2.A.56	1323	B, A, E
VIC	The voltage-gated ion channel superfamily	1.A.1	1122	B, A, E
MC	The mitochondrial carrier family	2.A.29	1051	E

[a] B, bacteria; A, archaea; E, eukaryotes.

families are grouped into superfamilies, which define the evolutionary relationships between individual families. TCDB also provides web-based search tools and various bioinformatic software packages that allow users to search by key word, gene name, family, or protein sequence. There is also a major section devoted to poorly characterized families of transport or putative transport proteins where more studies are needed.

TransportDB: A Comprehensive Database Resource for Transporters

TransportDB (http://www.membranetransport.org/) is a relational database surveys fully sequenced genomes for genes encoding transport proteins. It was created as a comprehensive database resource of information on cytoplasmic membrane transporters and outer membrane channels in organisms whose complete genome sequences are available. The complete set of membrane transport systems and outer membrane channels in each organism were annotated based on a series of experimental and bioinformatic evidence and classified into different types and families according to the TC system. User-friendly web interfaces were designed for easy access, query, and download of the data. Features of the TransportDB website include text-based and BLAST search tools against known transporter and outer membrane channel proteins; comparison of transporter and outer membrane channel contents from different organisms; known three-dimensional structures of transporters; and phylogenetic trees of transporter families. On individual protein pages, users can find detailed functional annotation, supporting bioinformatic evidence, protein/nucleotide sequences, publications, and cross-referenced external online resource links. One of the recent features added to TransportDB is the transporter automatic annotation pipeline web server where users can submit their genome or transporter annotation utilizing the high-throughput transporter analysis pipeline, view automatic annotation and supporting evidence, as well as curate the transporter annotation, all of which are through a user-friendly web interface.

TransportDB has now been in existence for over 10 years and continues to be regularly updated with new evidence and data from newly sequenced genomes as well as having new features added periodically. As of June 2007, TransportDB contains data from 289 species, including 232 bacteria, 24 archaea, and 33 eukaryotes. Over 90 000 transporter proteins from these organisms were identified and classified into 134 families, including 7 families of primary transporters, 80 families of secondary transporters, 32 channel protein families, 2 PTSs, and 13 unclassified families. Some of these transport protein families are very large superfamilies with thousands of members, such as the ATP-binding cassette (ABC) superfamily and the MFS, both of which are widely distributed in eubacteria, archaea, and eukaryotes (**Table 2**). Others are very small families with only a few members present

Table 2 Top organisms with highest percent of ABC superfamily transport proteins

Organism	*Number of ABC*	*Total*	*Percent of ORFs*
Agrobacterium tumefaciens C58	654	5402	12.1
Sinorhizobium meliloti 1021	593	6205	9.6
Thermotoga maritima MSB8	171	1858	9.2
Mesorhizobium loti MAFF303099	653	7275	9.0
Brucella melitensis 16M	279	3198	8.7
Brucella suis 1330	282	3264	8.6
Bordetella parapertussis 12822 NCTC-13253	360	4185	8.6
Bordetella bronchiseptica RB50 NCTC-13252	425	4994	8.5
Bifidobacterium longum NCC2705	140	1729	8.1
Streptococcus thermophilus CNRZ1066	154	1915	8.0
Streptococcus agalactiae NEM316	168	2094	8.0
Erwinia carotovora SCRI1043	358	4472	8.0
Bordetella pertussis Tohama I NCTC-13251	268	3447	7.8
Streptococcus mutans UAB159	152	1960	7.8
Streptococcus agalactiae 2603V/R	163	2124	7.7
Yersinia pseudotuberculosis IP32953	309	4038	7.7
Streptococcus pneumoniae TIGR4	160	2094	7.6
Treponema denticola ATCC35405	211	2767	7.6
Bradyrhizobium japonicum USDA110	634	8317	7.6
Silicibacter pomeroyi DSS-3	324	4252	7.6
Symbiobacterium thermophilum IAM14863	254	3337	7.6
Yersinia pestis KIM	310	4168	7.4
Roseobacter sp. TM1040	283	3864	7.3
Lactobacillus acidophilus NCFM	136	1864	7.3

in a narrow distribution of organisms. The following sections look in detail at some representative membrane transport families.

Major Solute Transporter Families

ABC Superfamily

The ABC transporter superfamily is characteristically one of the largest protein families found in the genomes of both prokaryotes and eukaryotes, with thousands of identified members. Most ABC transporters consist of four structural domains: two highly hydrophobic transmembrane domains (TMDs) that often contain six membrane spanning α-helices and two hydrophilic cytoplasmic ATP-binding domains that are responsible for ATP hydrolysis to drive the transport process. ABC import systems typically require an additional substrate-binding protein and are prokaryotic-specific. In Gram-negative bacteria, this ligand-specific binding protein is periplasmic, while it is an extracellular lipoprotein bound to the membrane in Gram-positive bacteria. These ligand-specific binding proteins confer specificity and high affinity for various substrates. Prokaryotic ABC transporters are involved in the import of a diverse spectrum of solutes, including inorganic anions and cations, carbohydrates, amino acids and peptides, and so on. Prokaryotic ABC transport systems usually carry integral membrane and ABC domains encoded by distinct genes, which are often organized together in gene operons or clusters. There are also a large group of ABC exporters in both prokaryotic and eukaryotic species involved in the extrusion of various drugs, metabolites, toxins, lipids, and signal molecules. In these systems, the TM and ABC domains are usually fused in different combinations.

ABC transporters are one of the major superfamilies of proteins, represented in all three kingdoms of life and have thus far been found in every organism sequenced. They are particularly abundant in prokaryotes, constituting about 0.7–12% of all ORFs (**Table 2**). A group of α-proteobacteria encode the highest number of ABC transporter proteins found thus far, for example, *Agrobacterium tumefaciens* encodes 654 ABC family proteins (12% of the total number of ORFs), *Mesorhizobium loti* (653, 9% ORFs), *Bradyrhizobium japonicum* (634, 8%), *Sinorhizobium meliloti* (593, 10%), and *Brucella suis* (282, 9%). The expansion of the ABC transporter family in these α-proteobacteria may reflect an organismal requirement for high-affinity transport, since ABC transporters typically show higher substrate affinities than most secondary transporters. Other organisms with a high percentage of ABC family transporters include (1) a group of organisms that lack a complete tricarboxylic acid (TCA) cycle and an electron transfer chain, and therefore obtain their metabolic energy from the fermentation of carbohydrates. These organisms include *Mycoplasma* spp., spirochetes, *Streptococcus* spp., *Lactobacillus* spp., *Tropheryma whipplei*, *Mycobacterium leprae*, *Thermoanaerobacter tengcongensis*, and *Thermotoga maritime*. They likely generate ATP as their primary source of energy, and therefore ABC transporters are most frequently used to drive nutrient uptake and maintain ion homeostasis. (2) A group of photosynthetic organisms with the ability to synthesize an ATP pool via photosynthesis, like *Synechocystis* sp., *Nostoc* sp., *Roseobacter* sp., *Jannaschia* sp., and *Thermosynechococcus elongatus*.

The maltose transport complex in *E. coli* and *Salmonella typhimurium* is one of the best characterized members of ABC superfamily and can serve as a general model for ABC importers. They share over 90% identical amino acid residues and the components of the complex have been demonstrated to be fully exchangeable. Starch from decomposing plant materials in the environment or the human gastrointestinal tract is one of the major sources of carbon and energy for heterotrophic bacteria and some archaea. The uptake of starch degradation products, maltose and maltodextrin, are mediated by the ABC family maltose transporter complex. It has been shown that the ABC family maltose transport systems are widely distributed among Gram-positive bacteria, Gram-negative bacteria, and archaea. In *E. coli*, it is composed of a periplasmic maltose-binding protein (MalE), a transmembrane complex made up of the MalF and MalG proteins, and two copies of the ATPase subunit, MalK. Transport of maltose is initiated by interaction of substrate-bound MalE with the periplasmic loops of MalFG, which induces a conformational change that results in ATP hydrolysis at the MalK subunits and eventually in the translocation of substrates. In Gram-negative bacteria, it requires an additional component, LamB (maltoporin) on the outer membrane to mediate the diffusion of maltose and maltodextrin into the periplasm. The genes encoding the maltose components are usually clustered into closely linked operons. However, the ATP-binding component is often missing, suggesting a single ATPase could function with several transporters with similar functions. For example, the ATPase, MsiK, can function in the uptake of maltose and cellobiose mediated by different ABC transporters.

The periplasmic substrate-binding protein MalE in *E. coli* can bind to a variety of α-1,4-linked oligoglucosides, including linear, cyclic, reduced, and oxidized maltodextrins. However, only a portion of the bound ligands are subsequently transported into the cell. The binding of *E. coli* MalE to substrates that are actively transported results in an endothermic reaction that is entropy driven, while the binding to nontransported substrates is an exothermic reaction. MalE has been crystallized in the presence of maltose or maltodextrin. Similar to other substrate-binding proteins, MalE consists of two symmetrical lobes, with a substrate-binding site positioned in a cleft in between. The MalE protein undergoes conformational changes involving the bending of a hinge that joins the two lobes. There is an open conformation in which the binding site is accessible to the substrate. The binding of substrate initiates a closed conformation in which the two lobes move toward each other and trap the substrate inside the cleft. Binding proteins have two roles in the transport: they are responsible for the high substrate specificity of transport and they stimulate the ATPase activity. MalG and MalF are membrane-integral subunits with 6–8 hydrophobic membrane-spanning helices. They are usually less conserved than the ATP-binding counterparts. The presence of long interhelical periplasmic loops in MalG and MalF represents a general feature of ABC transporters. MalK is an ATPase with nucleotide-binding domain (NBD) for the ABC transporters. It is located on the cytoplasmic side of the membrane. Two copies of MalK form a dimer and dissociate through ATP hydrolysis, processes which are believed to be crucial to the action of ABC transporters, including both importers and exporters. The NBDs of ABC transporters are highly conserved and recent structural studies have confirmed that a wide variety of NBDs share extremely similar structures, and that there is no clear distinction between importers and exporters in NBD structure. NBDs possess Walker A and B motifs that are characteristic of all P-loop ATPases, as well as a C-loop ('LSGGQ') signature motif, a D-loop ('SALD'), a Q-loop ('Q'), and an H-loop ('H'). The crystal structure of MalK has been determined as a dimer in both nucleotide-free and ATP-bound forms, making it possible to directly visualize the MalK dimer in different conformation states. The two NBDs of MalK dimerize upon the binding of ATP in a 'sandwich' pattern, with ATP molecules bound along the dimer interface, flanked by the Walker A motif of one subunit and the LSGGQ motif of the other subunit. Residues in these motifs are involved in extensive interactions with ATP and are required to form the ATPase active sites. MalK as well as several other bacterial sugar transporters share a distinct feature: they contain an additional C-terminal domain of about 135 residues called the regulatory domain, which mediates the subunit–subunit interactions and contribute substantially to the dimer interface. The regulatory domain of MalK also interacts with regulatory proteins like enzyme IIA^{Glu}, part of the glucose PTS, and the transcriptional regulator MalT. The C-terminal domains of MalK maintain their intersubunit contacts all the time, while the N-terminal NBDs of MalK are in close contact only in the ATP-bound state. The motion of MalK implied by these structures is tweezer-like. The regulatory domains represent the handle that holds the two halves together, and the Q loops are located at the tips of the tweezers where they can move apart or together.

The crystal structures of isolated NBDs, like MalK, provide a detailed picture of how these two ATP-binding components interact with each other. However, high-resolution structures of an intact bacterial ABC importer are needed to illustrate how the NBDs interact with the TMDs and how the TMDs interact with each other. This type of information is essential for our understanding of the molecular mechanism by which ATP hydrolysis is coupled to transport. The high-resolution structures of several intact bacterial transporters have been determined: the vitamin B12 transporter BtuCD in *E. coli*, a metal chelate importer HI1470/1 from *Haemophilus influenzae*, a drug exporter Sav1866 from *Staphylococcus aureus*, and, most recently, a molybdate importer $ModB_2C_2A$ from *Archaeoglobus fulgidus*.

The 3.1 Å crystal structure of a putative archaeal molybdate transporter ($ModB_2C_2$) in complex with its binding protein (ModA) has been reported (**Figure 3**). This structure involved a single ModA subunit with bound substrate attached to the external side of $ModB_2C_2$, which allows unidirectional transport into the cytoplasm. Each ModB subunit contains 6 hydrophobic transmembrane helices, which form a total of 12 transmembrane segments (TMSs) in the transporter. Unlike the multidrug transporter Sav1866, the vitamin B12 importer BtuCD, or the putative metal chelate importer HI1470/71, the ModB subunits present an inward-facing conformation: a large cavity is formed by the two subunits facing the cytoplasmic side, which narrows toward the external membrane boundary and is closed by a gate beneath the interface with the binding protein. This cavity, only accessible from the cytoplasm, is suggested to represent the translocation pathway. Compared to the molybdate importers, the multidrug exporter Sav1866, however, exhibits an outward-facing conformation of the TMDs with the cavity facing external membrane.

Similar to other NBDs, the ATPase ModC subunits contain the highly conserved P-loops and LSGGQ motifs involved in ATP binding and hydrolysis. They are oriented in a head-to-tail arrangement, with the P-loops of one subunit juxtaposed to the LSGGQ motif of the other. In the nucleotide-free open conformation state, these motifs are separated by a gap. Also, during the open conformation, the attached binding protein ModA aligns the substrate-binding cleft with the entrance to the translocation pathway. Binding of ATP triggers a closed conformation. The observed spacing of the P-loops and the LSGGQ motifs in ModC is consistent with biophysical studies of the MalK transporter in which the solvent accessibility to the fluorescently labeled ATP-binding sites is increased in the absence of ATP. The ModC–ModB interface transmits critical conformational changes, which links the ATP binding and hydrolysis to the transport process. Residues around the Q-loop of ModC primarily contribute to this interface, as observed in the

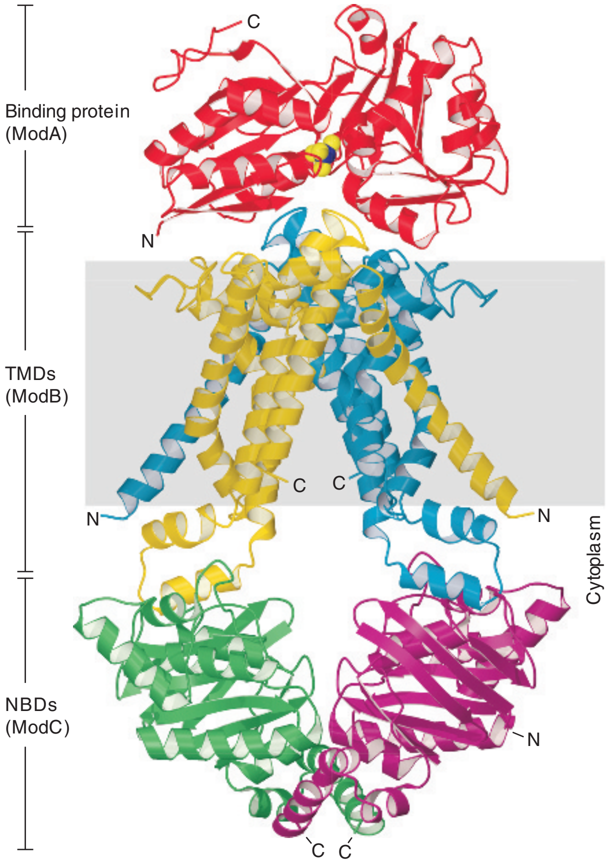

Figure 3 Front view of the $ModB_2C_2A$ complex in ribbon representation, with the ModB subunits colored yellow and blue, the ModC subunits colored green and magenta, the binding protein ModA colored red, and with bound tungstate in van der Waals representation (yellow and blue spheres). The gray box depicts the probable location of the lipid bilayer on the basis of the hydrophobicity of the protein surface. N, N-terminus; C, C-terminus. Note that there is a vertical twofold molecular and noncrystallographic symmetry axis for $ModB_2C_2$. Reproduced with permission from Macmillan Publishers Ltd. Hollenstein K, Frei DC, and Locher KP. Structure of an ABC transporter in complex with its binding protein. *Nature* 446(7132): 213–216.

structures of Sav1866, BtuCD, and HI1470/71. Structural comparison of $ModB_2C_2A$ with Sav1866 suggests a common mechanism for ATP-driven transport, with binding of ATP promoting an outward-facing conformation (as in Sav1866) and dissociation of the hydrolysis products promoting an inward-facing conformation (as in $ModB_2C_2A$). Binding of two ATP molecules at the interface of the NBDs closes the gap between the conserved ATP-binding motifs. As this gap closes, so does the distance between the attached coupling helices, causing the TMDs to flip from the inward-facing to the outward-facing conformation. This rearrangement may induce the conformation change of the attached binding protein ModA, which force the two lobes and releases the bound substrate. The substrate may diffuse from the binding site through the open gate and into the translocation pathway formed by the ModB subunits.

F-, V-, and A-Type ATPase

The proton-translocating F-, V-, and A-type ATPases are located in the cytoplasmic membranes of prokaryotes and the membrane of eukaryotic organelles, such as mitochondria and chloroplasts. It utilizes an electrochemical gradient of protons or sodium ions to synthesize ATP. It can also function in a reversible process in which an electrochemical gradient is established as the consequence of ATP hydrolysis. The bacterial F-, V-, and A-type ATPases consist of a water-soluble peripheral catalytic complex ($F_1/V_1/A_1$) with five subunits (alpha through epsilon) and an integral membrane proton translocation complex ($F_0/V_0/A_0$) with three subunits (subunits a–c). Synthesis or hydrolysis of ATP takes place in the catalytic complex, and is coupled to ion translocation across the single ion channel in the translocation complex via the rotation of a central part of the complex (rotor) relative to a static portion of the enzyme (stator) (**Figure 4**).

The F/V/A-ATPase family is widely distributed among archaea, eubacteria, and eukaryotes. This family can be further classified into three subfamilies: the F-type ATPases function mainly as an ATP synthase utilizing ADP and inorganic phosphate as substrate and is found on the plasma membrane of eubacteria, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts. The V(vacuolar)-type ATPases function

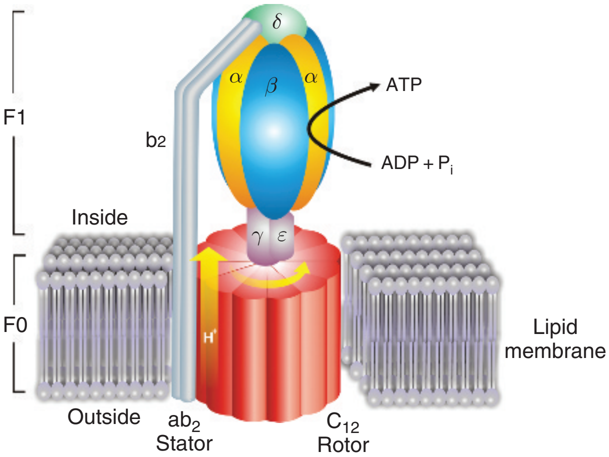

Figure 4 This diagram of ATP synthase within the mitochondrial inner membrane shows two major structural components F_0 and F_1 and the subunits within each. The F_0 portion consists of three subunits: a, b, and c. The two b-subunits firmly associate with the α- and β-subunits of F_1, holing them fixed to the membrane. The membrane-embedded cylinder of c-subunits is attached to the shaft of F_1. As protons move through the membrane via F_0, the cylinder and shaft rotate, and the β-subunits of F_1 change conformation as the γ-subunit associates with each in turn. See text for details.

exclusively as ATP hydrolysis-driven ion pumps and is found in the membranes of a wide variety of intracellular compartments, like chromaffin granules, lysosomes, endosomes, synaptic vesicles, Golgi-derived vesicles, the yeast vacuole, and the tonoplast of plants. They are involved in many intra- and intercellular processes, including receptor-mediated endocytosis, protein trafficking, pH maintenance, and neurotransmitter release. The A(archaeal)-type ATPases are found exclusively in archaea and can function in either direction.

The F-, V-, and A-type ATPases represent the smallest rotary motors found in living cells. Most of what we know about the structure and mechanism of these rotary molecules comes from studies on F-ATPase. Several crystal structures are available for F_1 complex of the F-type ATPase, including a 4.4 Å resolution X-ray crystallography structure from *E. coli*, and a 3.2 Å resolution structure from *Bacillus*. They all show a hexagonal barrel of alternating α- and β-subunits about 100 Å long and 120 Å wide. The α- and β-subunits display significant similarity at the level of both primary and tertiary structures. The hexagon of α- and β-subunits contains a central cavity, within which the γ-subunits locate. The middle portion of gamma bounds to the ε-subunit. Together they form a central stalk that protrudes from the bottom of α_3–β_3 hexagon. The γ- and ε-subunits also make contact with the membrane-embedded ring through the c-subunit of F_0. A peripheral stalk, made of a single copy of the δ-subunit (F_1) with two copies of b-subunits (F_0), connect the membrane-embedded ring through the a-subunit of F_0. Its role is to act as a stator to hold the catalytic α_3–β_3 hexagon and the a-subunit static relative to the rotary element of the enzyme, which consists of the c-ring in the membrane and the attached central stalk. The F_1 complex has six nucleotide-binding sites, all located at the interfaces of the α- and β-subunits and formed by amino acid residues from α- and β-subunits. There are three noncatalytic binding sites formed mainly by residues from α-subunits, with some contributions from the adjacent β-subunits. These sites are filled with magnesium nucleotide and show little variation. The function of the noncatalytic nucleotide-binding sites is not fully understood, but it is thought that they might be important for the assembly and stability of the complex. There are also three catalytic binding sites mainly composed of β-subunits, with some contribution from the adjacent α-subunits. These binding sites show variable states, with the binding of MgAMP-PNP, MgADP, or an empty state. Compared to the bound β-subunit, the structure of the empty β-subunit is significantly different: the nucleotide-binding pocket is collapsed and the entire C-terminal domain is rotated and shifted downward. Compared to the structurally well-characterized F_1 domain, there is no high-resolution structural model for the intact membrane F_0 domain of the F-ATPase. The c-subunits from different species are able to form rings of 10–14 proteolipids: the F_0 complex from *Ilyobacter tartaricus* has 11 proteolipids; the yeast enzyme has 10; and the chloroplast has 14. None of these structures contains the a-subunit, which has been modeled as a transmembrane protein with 5–6 transmembrane α-helices.

The current model of ATP synthesis by the F_0F_1-ATPase, known as the rotary catalytic model, was first proposed by Boyer and coworkers based on detailed analysis of the kinetics of F_1-ATPase activity (**Figure 4**). According to this model the F-, V-, A-type ATPase can be divided into a static portion (stator) and a mobile portion (rotor). In the *E. coli* F-ATPase, the stator is composed of α_3–β_3–δ–a–b_2 with a molecular weight of 380 kDa, and the rotor consists of γ–ε–c_{10} (150 kDa). The proton-motive force across the cell membrane, generated by the electron transport chain, drives the passage of protons through the membrane via the F_0 region of ATP synthase. The rotor (the ring of c-subunits) rotates as the protons pass through the membrane. The c-ring is tightly attached to the asymmetric central stalk consisting primarily of the γ-subunit which rotates within the stator (α_3–β_3 of F_1) causing the three catalytic nucleotide-binding sites to go through a series of conformational changes that leads to ATP synthesis. ADP and Pi (inorganic phosphate) spontaneously bind to the three β-subunits of the F_1 domain, so that each time it goes through a 120° rotation an ATP is released (rotational catalysis). The stator is prevented from rotating in sympathy with the central stalk rotor by a peripheral stalk that joins the α_3–β_3 to the nonrotating portion of F_0 (a-b_2). The structure of the intact ATP synthase is currently known at low resolution from electron cryomicroscopy studies of the bovine heart mitochondria complex. It shows that the peripheral stalk is a flexible rope-like structure that wraps around the complex and connects F_1 to F_0. It can serve as a stator during ATP synthesis and ATP hydrolysis.

Major Facilitator Superfamily

The MFS is an ancient, large, and diverse superfamily of secondary transporters, which use an electrochemical gradient to drive substrate translocation across the membrane. They catalyze uniport in which one type of solute is driven across the membrane by its own substrate gradient, symport in which two or more types of solutes are pumped in the same direction simultaneously utilizing the electrochemical gradient of one of the solutes as the driving force, and antiport in which solutes are transported in opposite directions across the membrane. Most transporters in this family have 400–600 amino acid residues and usually possess either 12 or 14 putative transmembrane α-helical segments. MFS permeases exhibit specificity for a diverse range of substrates, such as sugars, sugar phosphates, polyols, drugs, neurotransmitters, metabolites, amino acids, peptides, osmolites, iron siderophores, nucleosides, organic and inorganic anions, and so on.

The MFS transporters are found almost ubiquitously across all three kingdoms of living organisms. Like the ABC superfamily, MFS transporters are abundant in prokaryotes with more than 6000 members identified to date in sequenced prokaryotic genomes, constituting up to 3% of ORFs (**Table 3**). *Picrophilus torridus* encodes the highest percent of ORFs as MFS transporters (51, 3.3% of ORFs). Only certain obligate intracellular organisms with highly compact genomes do not encode MFS transporters, for example, *Phytoplasma asteris*, *Mesoplasma florum*, *Borrelia afzelii*, *Borrelia garinii*, *Treponema pallidum*, *T. whipplei*, *M. leprae*, and *Nanoarchaeum equitans*.

The high-resolution three-dimensional structures of the glycerol-3-P:P antiporter (GlpT) and the LacY have been determined. These structures reveal the twofold symmetry as expected, based on the sequence similarity of the two halves. The substrate pathway is predicted to exist between the two halves of the permeases using an alternating access mechanism with a single substrate-binding site. This mechanism is termed as the 'rocker switch' type of movement.

GlpT is a major *E. coli* MFS uptake system for glycerol-3-phosphate. It functions in the uptake of glycerol-3-phosphate through an antiport mechanism in which an inorganic phosphate is simultaneously exported from the cell. GlpT also catalyzes a reversible phosphate:phosphate exchange. Mutants that lack the GlpT system fail to exchange internal phosphate for either external phosphate or glycerol-3-phosphate. The K_m for the transport of glycerol-3-phosphate via GlpT was estimated to be $\sim$20 $\mu\text{mol}\,\text{l}^{-1}$. The crystal structure for GlpT has been determined at 3.3 Å. It reveals two domains connected by a long central loop. These N- and C-terminal domains, each containing a six-helix bundle, are related by a pseudo-twofold symmetry axis perpendicular to the membrane plane. The central loop linking the two domains is long, whereas most loops connecting the transmembrane α-helices of both domains are very short, leaving little freedom for relative movement of the helices within each domain. A substrate translocation pore is located between the two domains in an inward-facing conformation with pore open to the cytoplasm and closed to the periplasm. Two arginines at the closed end of the pore comprise the substrate-binding site for the negatively charged phosphate moiety. Upon phosphate binding to the arginines, GlpT adopts a more compact conformation, with the N- and C-terminal domains moved closer and the cytoplasmic pore narrowed. Substrate binding also destabilizes the interface between the N- and C-terminal domains on the periplasmic side and allows further tilting of the two domains to expose the substrate-binding site to the periplasm. In the periplasm, the lower affinity of the transporter for phosphate allows its replacement by glycerol-3-phosphate, whereas in the cytoplasm phosphate replaces glycerol-3-phosphate at the binding site due to its much higher cytosolic concentration.

The lactose permease LacY is a lactose/proton symporter, responsible for the uptake of lactose and other galactosides. The *E. coli* LacY is probably the best characterized secondary

Table 3 Top organisms with highest percent of MFS transport proteins

Organism	*Number of MFS transport proteins*	*Total ORFs*	*Percent of ORFs as MFS transport protein*
Picrophilus torridus DSM9790	51	1535	3.3
Thermoplasma acidophilum DSM1728	34	1478	2.3
Francisella tularensis WY96-3418	35	1634	2.1
Escherichia coli K12-MG1655	89	4237	2.1
Sulfolobus acidocaldarius DSM639	46	2223	2.1
Bacillus subtilis 168	84	4112	2.0
Oenococcus oeni MCW PSU-1	33	1691	2.0
Francisella tularensis Schu4	31	1603	1.9
Burkholderia pseudomallei K96243	106	5729	1.9
Rickettsia conorii Malish7	25	1374	1.8
Burkholderia mallei ATCC23344	84	4764	1.8
Rickettsia prowazekii MadridE	14	835	1.7
Salmonella typhimurium LT2	74	4451	1.7
Corynebacterium glutamicum ATCC13032	49	2993	1.6
Pseudomonas aeruginosa PAO1	88	5567	1.6
Sulfolobus tokodaii strain7	44	2826	1.6
Thermoplasma volcanium GSS1	47	3025	1.6
Bacillus anthracis A2012	86	5544	1.6
Coxiella burnetii RSA493	31	2009	1.5
Pseudomonas putida KT2440	82	5350	1.5
Pseudomonas fluorescens Pf-5	94	6137	1.5

transporter. The functional roles of every residue in LacY have been probed by Cys-scanning mutagenesis. LacY transports lactose and melibiose with similar affinities with a K_m ~0.1–1 mmol l^{-1}. The crystal structure of LacY has also been determined at 3.5 Å resolution. The structure of LacY was derived from a C154G mutant that is capable of binding substrate with high affinity, but catalyzes little or no transport. The structure was solved in the presence of the high-affinity lactose homologue β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (TDG). Residues that play major roles in substrate recognition and proton translocation were also identified. The LacY structure is highly similar to that of GlpT. It is composed of two pseudo-symmetrical bundles with six transmembrane helices each. Both structures reveal a large internal cavity containing bound sugar and open to the cytoplasm, but completely closed to the cytoplasm. The substrate-binding site is better characterized in LacY than in GlpT, largely due to the presence of substrate in the solved structure. The LacY structure, in complex with the substrate homologue TDG, shows the sugar bound within this hydrophilic cavity at a similar distance from either side of the membrane and in the vicinity of the approximate molecular axis of LacY. The two galactopyranosyl rings of TDG bind to the N- and C-terminal six-helix domains. The sugar-binding site is the N-terminal domain is composed of residues from helices I, IV, and V, while the helices VII and XI of the C-terminal domain, which are symmetrically related to helices I and V, form the other half of the binding site.

Based on the structure of LacY and GlpT and a large body of biochemical and biophysical evidence, a mechanism is proposed in which the transporter operates via a single binding site, alternating access model. The substrate-binding site is accessible from only one side of the membrane at a time. Initially, the substrate-binding site is accessible from the extracellular space in the outward-facing conformation. The binding of substrate to the binding site induces a conformational change that exposes the substrate-binding site to the cytoplasm in the inward-facing conformation for release. It is reasonable to think that other MFS transporters maintain a similar basic architecture and substitute key residues in the substrate-binding site to change the specificity of the transporter.

Dicarboxylate/Amino Acid:Cation Symporter Family

Transporters in the dicarboxylate/amino acid:cation symporter (DAACS) family mediate the symport of sodium ions or protons together with dicarboxylates like malate, succinate, and fumarate or various amino acids such as glutamate and aspartate. It is a much smaller transporter family compared to MFS, with 434 members identified in the TransportDB, but is also widely distributed in that data set, being present in 169 organisms across the three domains of life. *B. japonicum* and *Photobacterium profundum* possess the highest number of DAACS family transporters, each of which has nine.

The bacterial DAACS transporters are typically about 450 amino acid residues in length. Biochemical analyses of the topology of glutamate transporters, suggest that they have eight membrane-spanning α-helices. In addition, the C-terminal region contains two pore-loop structures, located between TMSs 6 and 7 and between TMSs 7 and 8 that partly reenter the membrane from opposite sides. The C-terminal region, including the reentrant loops and TMSs 7 and 8, has been a focus of study because it contains sites that interact with glutamate and competitive analogues, and with key ions such as sodium and potassium. The *E. coli* GltP is a proton-dependent transporter for glutamate and aspartate with K_m of ~5 μmol l^{-1}. It was shown to function as a homotrimer, and via an electrogenic symport mechanism where L-glutamate was cotransported with at least two protons. The mammalian glutamate transporters are typically about 550 residues in length. They catalyze the concentrative uptake of glutamate from the synapse to intracellular spaces utilizing preexisting ion gradients. These transporters are essential for normal development and function of the central nervous system and are implicated in stroke, epilepsy, and neurodegenerative diseases. Prokaryotic and eukaryotic glutamate and neutral amino acid transporters share significant amino acid sequence similarities throughout the entire polypeptide.

The three-dimensional structure of a member of the DAACS family Glt_{Ph}, a sodium ion-coupled aspartate transporter from the thermophilic archaeal species *Pyrococcus horikoshii*, has been determined to 3.5 Å. The Glt_{Ph} transporter structure was trimeric with three wedge-shaped subunits assembled to form a bowl-like structure. The basin faces the extracellular solution and the smaller base faces the cytoplasm. The deep, hydrophilic surface of the basin interior extends halfway across the membrane bilayer. An electron density consistent with a bound molecule of glutamate is located between the reentrant hairpin loops, and interacts with key amino acids in TMSs 7 and 8. The substrate-binding sites have been identified on each of the three subunits. It is proposed that the two hairpin loops serve as the gates controlling intracellular and extracellular access, respectively. At least three conformational states are involved in the transport cycle: open to the outside, occluded, and open to the inside. The transport of glutamate is achieved by movements of the hairpins that allow alternating access to either side of the membrane. Two sodium ions bind to TMS 7 and in close proximity to the substrate. These sodium-binding sites, together with the TMS 7 α-helix function as the central element of the ion-binding motif, which participates in conformational changes between the different states during the transport cycle. This mechanism is quite distinct from the 'rocker switch' mechanism, suggested by the studies of the structures of MFS transporters.

Phosphotransferase System

The PEP:sugar PTS is a complex enzyme system functioning in the detection, transport, and phosphorylation of various sugar substrates, including monosaccharides, disaccharides, amino sugars, polyols, and other sugar derivatives. The PTS family transporters use PEP as the energy source and the phosphoryl donor to carry out their catalytic function in sugar transport and phosphorylation. The basic components of the PTS are similar in all species studied. It is comprised of two general cytoplasmic components, enzyme I (EI) and phosphoryl carrier protein (HPr), which are common to all PTS carbohydrate transporters. The multidomain enzyme II (EII) complex is sugar-specific. Eubacteria usually encode many different EIIs. Each EII complex consists of one or two hydrophobic integral membrane domains (domains C and D) and two hydrophilic domains (domains A and B). The three or four domains together are responsible for the transport of the carbohydrate across the bacterial membrane as well as its phosphorylation. EII complexes can be formed by either distinct proteins or a single multidomain protein. Likewise, fusion proteins that contain EI and/or HPr domains exist. A prominent example of the latter is FPr, which consists of HPr and an EIIA domain and mediates phosphoryl transfer during the uptake of fructose by *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Rhodobacter capsulatus*. The PTS transporters are found exclusively in Gram-positive and Gram-negative eubacteria and are absent in archaea and eukaryotes. *Enterococcus faecalis*, *Lactobacillus plantarum*, and *Listeria monocytogenes* exhibit the highest number of PTS systems among all the sequenced prokaryotic genomes, each have about 40 EII complexes. About 15 different EII complexes are encoded in *E. coli* and *Bacillus subtilis*. The properties of these enzymes have been established by various genetic, biochemical, and physiological studies. Paralogues of EI and HPr have also been identified in some species. For example, five paralogues of each general PTS protein were discovered in *E. coli*.

In a typical phosphoryl transfer reaction (**Figure 5**), EI is phosphorylated using a phosphoryl group from PEP first, which initiates a chain of reactions. Then phosphohistidyl-EI phosphorylates a histidyl residue in HPr. This phosphorylation then transfers its phosphoryl group to a histidyl residue in EIIA of the sugar-specific EII complex. The phosphoryl group is then sequentially transferred to EIIB, and is finally transferred to the transported sugar bound to the membrane components, EIIC and/or EIID. All the phosphoryl derivatives in this multistep phosphoryl transfer system exhibit similar energy. The phosphorylation occurs at either histidyl or cysteyl residues.

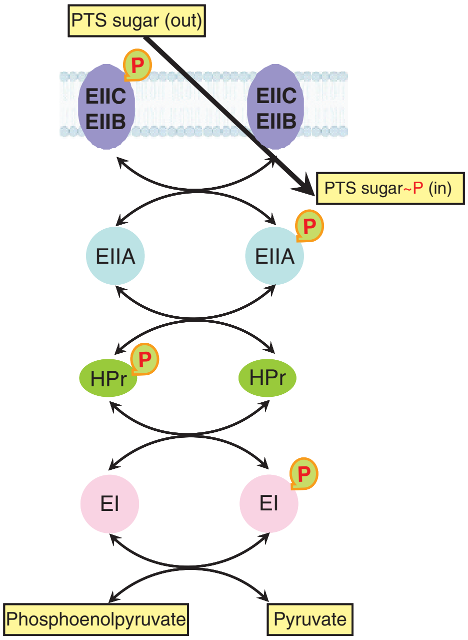

Figure 5 General phosphoryl and sugar transport reaction catalyzed by the PTS. Sugars are transported and concomitantly phosphorylated by the PTS. See text for details.

E. coli EI is a 63 kDa protein which contains about 570 residues and is encoded by the *ptsI* gene. Sequence comparisons reveal significant similarities among EIs from various Gram-positive and Gram-negative eubacteria. EI is autophosphorylated in the presence of Mg^{2+} at the N-3 position of the imidazole ring of conserved histidine residue on the N-terminus of the protein, which also contains the binding site for HPr. The C-terminus of EI contains the PEP-binding site and is required for dimerization. The structure of a full-length EI of *Staphylococcus carnosus* reveals that the N-terminal phosphohistidine and HPr-binding domain are clearly separated from the C-terminal dimerization and PEP-binding domain. EI forms a homodimer that accepts the phosphoryl group from PEP. The inactive monomeric EI exhibits a relatively high structural variability. The dimerization and the binding of Mg^{2+} and PEP induce conformational changes that bring the C-terminal domain with the two bound ligands close to the active site, which is necessary to facilitate the phosphotransfer.

HPr is a small, heat-stable protein (about 90 residues, 9–10 kDa). It is encoded by the *ptsH* gene. HPr is phosphorylated at the N-1 position of the imidazole ring of a histidyl residue (His-15) by PEP and EI. In most low-GC Gram-positive eubacteria and a few Gram-negative organisms, HPr can also be phosphorylated by a regulatory ATP-dependent Hpr(Ser) kinase on a seryl residue (Ser-46). This is not part of the phosphoryl transfer to carbohydrates, but phosphorylation of the seryl residue slows the phosphoryl transfer from EI to HPr at least 100-fold. *E. coli* HPr contains an Ser-46 residue but lacks an HPr(Ser) kinase. However, replacement of Ser-46 by an aspartyl residue significantly lowers the affinity of EI for HPr. The three-dimensional structure of HPrs from *E. coli*, *B. subtilis*, and several other species are known. It forms an open-faced β-sandwich, which consist of four antiparallel β-sheets that is covered at one side by one short and two long α-helices. The active histidyl residue (His-15) is located in the N-terminal part of the first long α-helix and is exposed to the solvent. The β-sheet curls back on itself so that the regulatory seryl residue (Ser-46) is close to the active histidyl residue. Thus, the presence of the negatively charged phosphoryl group at Ser-46 may inhibit the phosphorylation at the active site by electrostatic repulsion or by inhibition of EI binding.

The carbohydrate specificity of the PTS is dependent on the EII complexes, which consist of integral membrane domains/proteins (EIIC/EIID) and cytoplasmic domains/proteins (EIIA/EIIB). The PTSs were classified into four superfamilies with distinct evolutionary origins on the basis of the phylogenies of the EII complexes: (1) the glucose–fructose–lactose superfamily, comprised of the glucose family (TC 4.A.1), the fructose–mannitol family (TC 4.A.2), the lactose family (TC 4.A.3), and the glucitol family (TC 4.A.4); (2) the mannose family (TC 4.A.6); (3) the ascorbate–galactitol superfamily, comprised of the ascorbate family (TC 4.A.7) and the galactitol family (TC 4.A.5); and (4) the dihydroxyacetone (DHA) family. The sequence-based classification of the various PTSs is supported by X-ray crystallography and nuclear magnetic resonance studies, which clearly show that the structures of the EIIA and EIIB domains/proteins belonging to the various classes are quite different. Information on the structure of the integral membrane domain EIIC (and EIID) is limited. A large-scale bioinformatic study of the membrane topologies of EIIC in the glucose–fructose–lactose superfamily, suggest that members of this superfamily exhibit similar average hydropathy plots with eight TMSs α-helices and two reentry loops located between TMSs 6 and 7 and TMSs 7 and 8, respectively. The regions of average amphipathicity and relative conservation are also similar among all these members.

PTS also carries out numerous regulatory functions. The four proteins/domains (EI, HPr, EIIA, and EIIB) form a PTS phosphorylation cascade that can phosphorylate or interact with numerous non-PTS proteins and thereby regulate their activity. PTS regulation network not only controls carbohydrate uptake and metabolism but also interferes with the utilization of nitrogen and phosphorus and the virulence of certain pathogens. In *E. coli*, nitrogen enzyme I ($\mathrm{EI^{Ntr}}$), nitrogen HPr (NPr), and nitrogen IIA protein ($\mathrm{IIA^{Ntr}}$), paralogues of EI, HPr, and $\mathrm{EIIA^{Fru}}$, respectively, constitute a phosphoryl transfer chain that has been shown to exhibit little enzymatic cross-reactivity with the classical sugar-transporting phosphoryl transfer chain consisting of EI, HPr, and various sugar-specific EII complexes. This nitrogen-related phosphoryl transfer chain presumably functions only in regulation. $\mathrm{EI^{Ntr}}$ homologues have been shown to cluster phylogenetically together, distantly from all other EI homologues. $\mathrm{EI^{Ntr}}$ may serve a sensory function linking carbon and nitrogen metabolism. A mutation of the $\mathrm{EI^{Ntr}}$ gene resulted in impaired metabolism of poly-β-hydroxybutyrate, and diminished respiratory protection of nitrogenase under carbon-limiting conditions. In addition to $\mathrm{EI^{Ntr}}$ and NPr, *E. coli* encodes within its genome three additional EI paralogues and four additional HPr paralogues. The functions of most of these proteins are still unknown. A nontransporting EII complex was characterized in *E. coli* recently. It phosphorylates DHA at the expense of PEP, using three soluble DHA-specific proteins (DhaK, DhaL, and DhaM), in addition to EI and HPr. The DhaM protein consists of three domains: an N-terminal $\mathrm{IIA^{Dha}}$ domain that is distantly related to $\mathrm{IIA^{Man}}$, a central HPr domain, and a C-terminally truncated EI domain. All three domains can be phosphorylated by PEP with the EI and HPr as phosphoryl donors.

Comparative Studies of Transporter Family Distribution Show Strong Influence of Physiology and the Living Environment

Recent advancements in genome sequencing make it possible for the comparative analyses of essential cellular processes like transport in organisms across the three domains of life. As of December 2007, over 600 prokaryotic genomes have been sequenced and deposited in the public databases. These genomes cover a broad range of microbial organisms from different phylogenetic groupings, allowing comparative genomic analyses across a diverse range of organisms and lifestyles. A recent study of phylogenetic profiles of transporter families, which are derived from the presence or absence of a certain transporter family, showed that organisms that clustered together based on similar transporter distribution patterns appeared to reflect both phylogenetic and environmental factors. For instance, three large groups of organisms with similar transporter profiles were identified that appeared to reflect their physiology and living environment: obligate intracellular organisms, plant/soil-associated organisms, and autotrophs. Each of these groups exhibited distinct patterns of transporter family distribution. The obligate intracellular organisms possessed the fewest types and number of transporters, while plant/soil-associated organisms generally encoded the largest variety and number of transporters. The cluster of autotrophic organisms generally lacked transporters for carbohydrate and organic nutrients, while possessing a range of transporters for inorganic ions and molecules.

Obligate Intracellular Pathogens/ Endosymbionts

The cluster of obligate intracellular organisms includes a group of phylogenetically diverse intracellular pathogens or endosymbionts, including Chlamydia (pathogens); γ-proteobacteria like *Buchnera* spp., *Wigglesworthia glossinidia*, and *Candidatus Blochmannia* spp. (endosymbionts); α-proteobacteria such as *Wolbachia* spp. (endosymbionts) and *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Neorickettsia sennetsu*, *Bartonella* spp. (pathogens); low-GC Gram-positive-like organisms *Mycoplasma* spp., *Ureaplasma urealyticum*, *P. asteris*, and *T. whipplei* (pathogens); spirochetes like *T. pallidum*, *Borrelia* spp. (pathogens); and an archaeal endosymbiont, *N. equitans*. Owing to the less dynamic nature of their intracellular environments, the transport requirements for these obligate intracellular organisms are probably more specialized than those of environmental organisms. This may have allowed them to shed, for example, transporters for alternative nitrogen/carbon sources, drug/toxic metabolite efflux, osmoregulation, and ion homeostasis. The residual transport systems conserved in these obligate intracellular organisms probably belong to the core essential genes required for the acquisition of key nutrients and metabolic intermediates. For example, in *Rickettsia* species, genes coding for proteins functioning in glycolysis and the biosynthesis of *S*-adenosylmethionine (SAM) and nucleotides are absent. They completely rely on the hosts for these small molecules. As expected, transporter systems for the uptake of nucleoside monophosphates (ATP:ADP antiporter family), SAM (drug/metabolite transporter family), and glycerol-3-phosphate (MFS family) have been identified. The essential glutamate transporter in two obligate endosymbionts, *Blochmannia floridanus* and *W. glossinidia*, provides another example: the GltP glutamate:proton symporter (DAACS family) is encoded in *B. floridanus*, while the GltJKL ABC transporter is expressed in *W. glossinidia*. Both of these organisms have a truncated TCA cycle which begins with α-ketoglutarate and ends with oxaloactetate. Their TCA cycle could be closed by the transamination of the imported glutamate to aspartate, catalyzed by an aspartate aminotransferase (AspC) which uses oxaloactetate as a cosubstrate and produces α-ketoglutarate. Therefore, two different transporters in two species use the same strategy to feed in the essential metabolite intermediates and fulfill the same metabolic goal. The genome sequencing of *Baumannia cicadellinicola*, an intracellular symbiont in the glassy-winged sharpshooter (*Homalodisca coagulata*), reveals that *B. cicadellinicola* encodes a very limited set of amino acid synthesis pathways. Except for histidine, no complete pathways for the synthesis of any essential amino acids are present. The lack of amino acid synthesis pathways is apparently compensated by the ability to import amino acids from the host using a general amino acid ABC transporter, an arginine/lysine ABC transporter, a lysine permease, and a proton/sodium-glutamate symporter.

Compared to species in other clusters, obligate intracellular organisms show a higher degree of variation in terms of energy-coupling mechanism and transport mode. These variations may reflect the unique internal environment inside the host cells. All these observations illustrate how adaptation of an organism to certain living conditions leads to changes in its transporter repertoire and at the same time determines the set of transporters that the organism cannot afford to lose. Another distinct feature of obligate intracellular organisms is the lack of a group of sodium ion-dependent transporter families, including the neurotransmitter:sodium symporter, alanine/glycine:cation symporter, solute:sodium symporter, and divalent anion:sodium symporter. Transporters in these families are all symporters which utilize the sodium ion gradient to transport amino acid, solute, and/or divalent ions to the cytoplasm. In general, free-living environmental organisms frequently encode a variety of sodium-dependent pumps. In contrast, obligate intracellular organisms have virtually completely lost these families, probably related to the very low sodium ion concentrations likely inside their host cells.

Soil/Plant-Associated Microbes

The cluster of soil/plant-associated microbes include organisms from various phylogenetic groups, for example, Actinobacteria (*Corynebacterium* spp., *Nocardia farcinica*, and *Streptomyces* spp.), γ-proteobacteria (*Actinobacter* sp., *Idiomarina loihiensis*, *Pseudomonas* spp., *Pseudoalteromonas haloplanktis*, and *Rhodopseudomonas palustris*), β-proteobacteria (*Bordetella* spp., *Burkholderia*, and *Ralstonia*), α-proteobacteria (*A. tumefaciens*, *Brucella* spp., *Jannaschia* sp., *M. loti*, *Silicibacter pomeroyi*, and *S. meliloti*), δ-proteobacteria (*Geobacter sulfurreducens* and *Pelobacter carbinolicus*), and ϵ-proteobacteria (*Wolinella succinogenes*). All of the organisms in this group possess a robust collection of transporter systems. The similarity of phylogenetic profiles of organisms in this cluster probably reflects the versatility of these organisms and their exposure to a wide range of different substrates in their natural environment. The majority of species in this cluster can be free living in the soil and some are capable of living in a diverse range of environments. They generally share a broad range of transport capabilities for plant-derived compounds, in particular, and for organic nutrients, in general. Interestingly, some human facultative pathogens, such as *Bordetella* and *Brucella*, show similar transporter family profiles with organisms in this group. These particular pathogens have close relatives that are soil- or plant-associated environmental organisms, so their transport capabilities probably reflect a combination of their evolutionary heritage, original environmental niche, and their current transport needs.

Autotrophs

The cluster of autotrophic organisms with similar transporter distribution profiles includes both obligate and facultative autotrophs. Obligate autotrophs obtain energy exclusively by the oxidation of inorganic substrates and use carbon dioxide as the only resource of carbon, such as the nitrifying bacteria *Nitrobacter winogradskyi* (oxidizing nitrite ion), and *Nitrosomonas europaea* and *Nitrosococcus oceani* (oxidizing ammonium ion). Facultative autotrophs obtain some part of their energy from oxidation of iron, sulfur, hydrogen, nitrogen, and carbon monoxide. These include green sulfur bacteria, (*Chlorobium* spp. and *Pelodictyon luteolum*) and green nonsulfur bacteria (*Dehalococcoides* spp.), both of which are anaerobic photosynthetic bacteria; Cyanobacteria (*Prochlorococcus* spp., *Synechococcus* spp., *Synechocystis* sp., *Nostoc* sp., *Gloeobacter violaceus*, and *T. elongatus*), which are aerobic photosynthetic bacteria; a hydrogen-oxidizing microaerophilic, obligate chemolithoautotrophs (*Aquifex aeolicus*); an obligate methanotroph, *Methylococcus capsulatus*; and a group of autotrophic archaeal species (*Aeropyrum pernix*, *Sulfolobus* spp., *P. torridus*, *Thermoplasma* spp., *Methanobacterium thermoautotrophicum*, *Methanopyrus kandleri*, *Methanococcus jannaschii*, *Pyrobaculum aerophilum*, *Pyrococcus* spp., *Thermococcus kodakaraensis*, *Natronomonas pharaonis*, *Haloarcula marismortui*, and *Halobacterium* sp.). In line with their metabolic features, organisms in this group generally lack transporters for carbohydrates, amino acids, carboxylates, nucleosides, and so on. Instead, they encode a full array of transporters for various cations and anions, ammonium, inorganic phosphate, and sulfate, which feed into their autotrophic metabolism. Transporter families for inorganic ions and small compounds are heavily represented in these autotrophs, including potassium and chloride ion channels (voltage-gated ion channel superfamily and chloride channel family), ammonium transporter, inorganic phosphate transporter, sulfate permease, and calcium:cation antiporter. These features distinguish this group of autotrophs from organisms in the plant/soil-associated and intracellular pathogen/endosymbiont clusters. Interestingly, some heterotrophic bacteria exhibit similar transporter profile to obligate autotrophs. They generally fall into several categories: pathogens that are evolved from environmental organisms, like *Leifsonia xyli* and *Leptospira interrogans*; organisms with extensive ion transport systems and/or few organic nutrient transporters, like *T. tengcongensis*, *Coxiella burnetii*, and *Mycobacterium* spp.; and a Thermotogales (*Thermotoga maritima*) with extensive array of archaeal-lineage genes, and was found to cluster with the archaeal species in this supercluster.

Conclusion

The era of structural biology and genomics has opened new horizons in our understanding of complex biological questions. The three-dimensional transporter structures have provided us invaluable information on the mechanisms of transporter function, while the comparative genomic approaches for the analysis of membrane transport systems rendered insights on how microbes adapt to their environment. The observations that organisms with similar lifestyles and/or ecologic niches (obligate intracellular, soil/plant-associated, or autotrophic) display similar phylogenetic profiles despite their phylogenetic differences strongly suggest the influence of living environment on organisms' membrane transport gene complement.

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Relevant Website

http://www.membranetransport.org/ – TransportDB

Transposable Elements

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The Advent of *In Vitro* Transposition Systems
Genome-Wide Knockout Analyses
Transposable Element-Based Deletion Studies
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Glossary

bacteriophage A virus that infects bacteria.

CFP Cyan fluorescent protein.

donor DNA The DNA on either side of the transposon from which the transposon moves during transposition.

recognition end sequences The short DNA sequences that define the two ends of a DNA transposon. Recognition end sequences are recognized by the transposon-specific transposase protein.

synapsis The formation of a transposase–transposon DNA dimeric complex, t is a required intermediate in transposition.

target DNA The DNA into which the transposon DNA is inserted following transposition.

Tn5 Transposon 5, a particular DNA transposon.

transposase The protein that catalyzes DNA transposition.

transposition The process by which a defined DNA sequence moves from one site in the genome to a second site.

transposon The defined DNA sequence that is moved as a consequence of transposition.

YFP Yellow fluorescent protein.

Abbreviations

CFP	cyan fluorescent protein
ER	end recognition
YFP	yellow fluorescent protein

Defining Statement

Transposons are powerful molecular genetic tools for performing both genome-wide genetic analyses and studies targeted to particular genes or gene regions. In addition to being amenable to standard genetic analyses, transposon technologies are important adjuncts to modern high-throughput techniques.

Introduction

The first use of transposable elements (to be called transposons for the remainder of this article) as tools for microbial genetics dates from the discovery of bacteriophage Mu by AL Taylor over 40 years ago. Taylor's initial report on Mu described how one could generate a wide variety of auxotrophic mutations merely by creating Mu lysogens. The frequency and variety of these mutations within a lysogen population suggested that all lysogens were mutants in which the phage genome had inserted into one of a wide variety of bacterial genomic sites. In other words, Taylor had discovered a biologically based mutagen that was incredibly efficient, apparently random, and very easy to use.

It was this observation by Taylor, bolstered by discussions of Mu's properties at Cold Spring Harbor Phage Meetings, that led various investigators to adopt Mu as a genome-scanning, knockout, mutagen. My own adoption of this tool, soon after I arrived at the University of Wisconsin–Madison as a new faculty member, was initiated by my attempt to use Mu to identify positive regulatory genes for the *Escherichia coli* tryptophan operon. I used a *trp-lac* fusion strain and looked for Lac$^-$ Mu lysogens on Lactose-MacConkey agar. As it frequently happens in genetics, the selection/screen worked to yield interesting mutations (in the gene encoding phosphoglucose isomerase), but not in the mythical gene that I had imagined to exist. Nonetheless, the power of transposons as genetic tools was not lost on me and many other investigators.

The widespread acceptance of transposons as genetic tools in the *E. coli* and *Salmonella typhimurium* research communities followed from the discovery of two other types of transposons. An analysis of spontaneous knockout mutations in *E. coli* indicated that many of them were caused by the insertion of identical DNA sequences. These became to be known as IS elements, each type encoding its own transposase and containing specific terminal sequences that were recognized by the cognate transposase at the initiation of transposition. A second related class of mobile genetic elements was discovered in the mid-1970s. These elements were antibiotic resistance-encoding transposons that could move from one replicon (for instance, an antibiotic resistance-encoding plasmid called an R factor) to a second replicon (such as bacteriophage λ). I directly benefited from these discoveries as Jim Shapiro (one of the discoverers of IS elements) and I were postdoctoral fellows together shortly after his discovery of IS-associated mutations, and the next step in my research career led me to a laboratory adjacent to that of Julian Davies, who shortly thereafter discovered the antibiotic resistance-encoding transposon Tn5.

The discoveries of IS elements and antibiotic resistance-encoding transposons were coupled with the then 'new' recombinant DNA techniques to enable the structural dissection, modification, and genetic analysis of several transposons. A generic view of transposon structure is presented in **Figure 1**. An important result of these studies was the detailed determination of the key steps in the transposition process for several transposons (**Figure 1** also presents a schematic of transposition through one well-studied mechanism, 'cut and paste' transposition).

Molecular Participants in Transposition

The key molecular participants in 'cut and paste' transposition (see **Figure 1**) are quite simple. As described below, these participants include transposon-specific transposase, transposon DNA defined by recognition end sequences, target DNA, and Mg^{2+}.

1. *Transposase* For each type of transposon, there exists an element-specific protein called a transposase, which catalyzes the biochemical steps in transposition. For many frequently used transposons, the transposase is the only required protein although in some cases host proteins or additional transposon-encoded proteins are required.
2. *Recognition end sequences (defining the transposon structure)* Each transposon end is defined by a specific DNA recognition end sequence to which the transposase specifically binds to initiate the transposition process. For some transposons, these ends are as short as 18 bp. If the transposase is encoded elsewhere, the content and length of the DNA found between the two recognition end sequences can be anything, provided it is not too short (a 256-bp Tn*5* derivative has been successfully used) or too long as to prevent the formation of transposition intermediates (see **Figure 1**).
3. *Target DNA* The final step in transposition involves the integration of the transposon DNA into the target DNA sequence on the same or on a different DNA molecule (**Figure 1**). Many transposons are rather random in their target sequence choice with the exact target primarily chosen based on the random collision between the excised element and the target. However, there are some sequence biases that have been determined for those transposons that have been carefully studied.
4. *Mg^{2+} (or Mn^{2+})* The divalent cation Mg^{2+} plays an absolutely key role in the catalytic steps for transposition.

Transposon Content

The realization that transposon DNA is flexible in content and the use of recombinant DNA technology to modify the content of transposons have been important steps in developing transposons as true tools in molecular genetic analyses, making them far more than mere knockout mutagenesis agents. In essence, anything that the investigator can conceive of with regard to a desired DNA sequence can be incorporated between the two recognition end sequences. General categories of interesting DNA sequences to consider include the following.

1. *Selectable functions* The fundamental genetic information that is needed within a transposon for almost all applications is a selectable marker. In the original Mu work of Taylor, the selectable marker was immunity to Mu superinfection. Transposons in use today typically encode resistance to one or more antibiotics. The architects of these antibiotic-resistant transposons have merely borrowed from or copied the transposons that were discovered in the 1970s.
2. *Reporter functions* A variety of reporter functions have been included in the body of transposons typically with the reporter gene abutting one recognition end sequence. In operon fusion systems, the reporter gene is complete with its own translation initiation signals, but lacks a transcription initiation signal between the reporter gene and the upstream end of the element. With this type of construct, the reporter gene expression will be driven by transcription that reads from the DNA adjacent to the target site and provides a qualitative measure of the level of the resulting fusion mRNA synthesis. Gene fusion reporter systems fuse an N-terminal truncated protein encoded by the transposon to a protein encoded by the target sequence. Thus, the fusion can be used to tag the target protein for determining its subcellular localization and to evaluate the level of transcription and translation of the

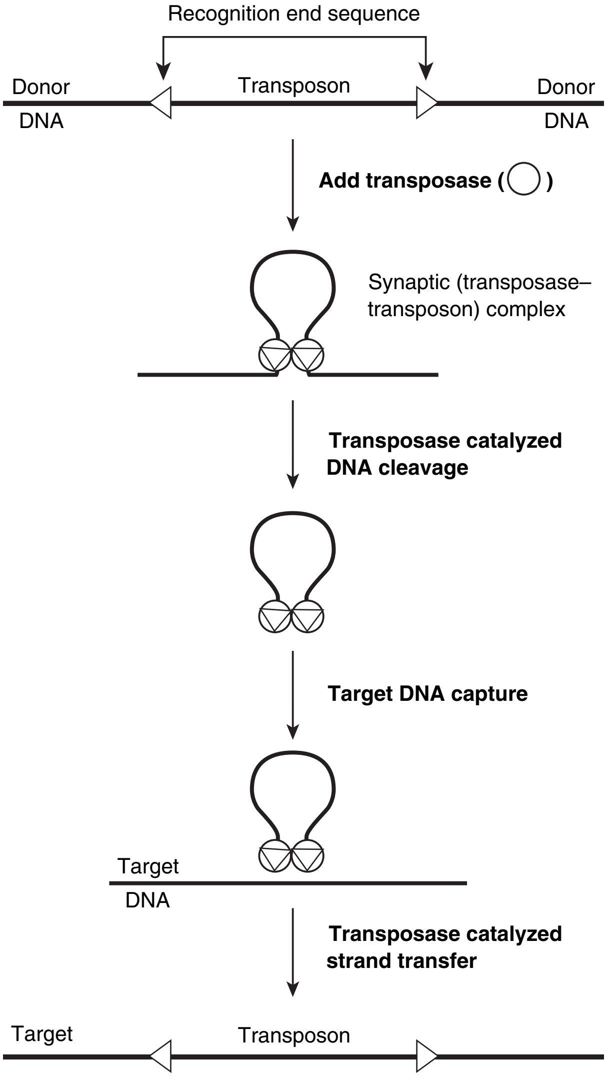

Recognition end sequence
Donor
DNA
Transposon
Donor
DNA
Add transposase ( )
Synaptic (transposase–transposon) complex
Transposase catalyzed DNA cleavage
Target DNA capture
Target
DNA
Transposase catalyzed strand transfer
Target
Transposon

Figure 1 Transposon structure and 'cut and paste' DNA transposition. DNA transposons are DNA sequences that are defined by specific end sequences (represented by two open triangles). Typically, a natural transposon contains a gene that encodes the specific transposase (represented by an open circle) that catalyzes transposition after binding to the specific recognition end sequences. However, if the transposase is supplied from somewhere else, the DNA between the specific end sequences can contain a wide variety of other genetic information. The figure additionally presents a simplified schematic of 'cut and paste' DNA transposition. The transposase binds to the recognition end sequences to form a synaptic complex. In the presences of Mg^{2+}, the transposase in the synaptic complex catalyzes cleavage of the DNA between the recognition end sequences and the donor DNA, thus releasing the transposition complexes. The transposition complexes then bind to target DNA and the transposase then catalyzes strand transfer, thus inserting the transposon into the target. Further details on transposon structure and the 'cut and paste' transposition mechanism can be found in Nancy Craig and Williams Reznikoff.

targeted gene. Typical reporter genes include those that encode β-galactosidase, alkaline phosphatase, and various fluorescent proteins. A specific example of the use of fluorescent protein fusions will be given later.

3. *Landmarks* Transposons by their very nature represent landmarks. That is, their integration inserts a recognizable DNA sequence within the target DNA and their locations and orientations can be easily mapped against other known genome locations. In addition, transposons have been designed to carry specialized landmarks. An obvious class of landmarks carried by all transposons is a primer-binding site that can be used for sequencing adjacent target DNAs. It is for this reason that transposons are frequently used as powerful DNA sequencing tools. Transposons have been designed to carry sequences that are targets for site-specific recombination systems (such as a P1 *lox* site, a λ *att* site, or an FRT site) so that additional genetic information can be subsequently incorporated or

unwanted transposon sequences can be removed. Rare cleavage sites can be included for physical mapping of insert locations or as counterselectable markers against the presence of the transposon or to allow the removal of unwanted transposon-encoded sequences (see below for an example of such a sequence removal process). Finally, transposons that carry multiple copies of *lac*O or *tet*O can be located through fluorescence microscopy within a cell or on long DNAs using cognate repressors fused to GFP derivatives (for instance, *Lac*I-CFP and TetR-YFP).

An interesting class of landmarks, which can be derived from inserted transposons, are sequences that encode specific amino acid residues in the interrupted target gene product. For instance, the encoded sequence may involve the insertion of a unique protease-sensitive site or a particular epitope. In general, these types of insertion products are examples of linker-scanning mutations. Typical transposons that are used to generate these insertions include a translation-fusion reporter function so that only insertions in the desired orientation and in the correct reading frame are chosen for subsequent analysis. They also have modified recognition end sequences so that they can encode the desired peptide-encoding sequence and there are site-specific recombination sites or rare restriction sites that allow the simple excision of the bulk of the transposon. Following excision, an insert containing $N \times 3$ nucleotides encoding the desired sequence is left behind within the target-encoded protein (**Figure 2**). In some cases, the modification is even more detailed, for instance, the resulting protein can have a single amino acid insertion, substitution, or deletion. Further examples will be presented subsequently.

4. *Controlling elements* Controlling elements are *cis* active DNA (or RNA) sequences through which the inserted transposon can direct adjoining DNA (or RNA) activities. Examples of controlling elements include the following:
   a. Promoter sequences for directing the transcription of adjacent DNAs.
   b. Transcription termination sequences to insure that the inserted transposon shall have a polar knockout effect on downstream genes.
   c. Origins of replication that will allow the inserted transposon to act (in conjunction with restriction

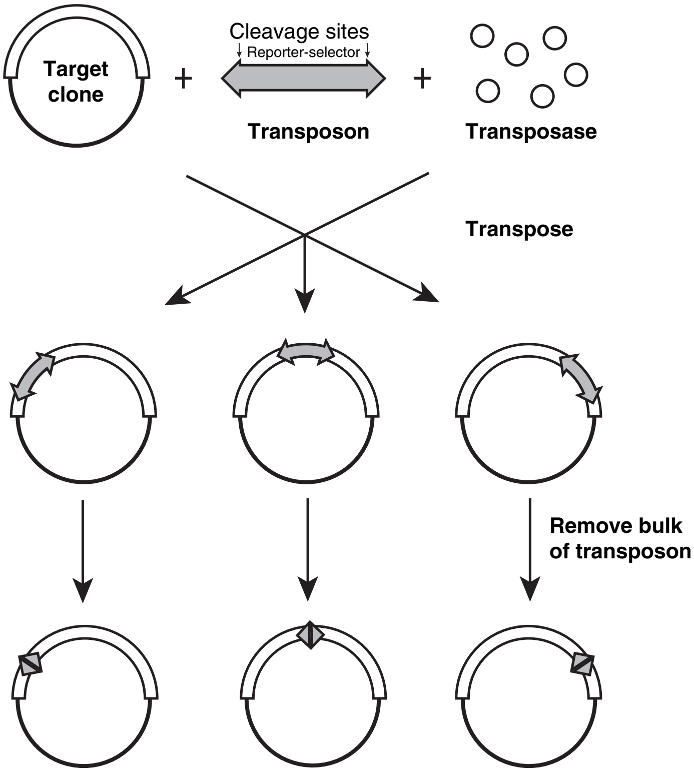


Figure 2 Transposons and the generation of linker-scanning mutations. Transposons can be used to generate linker-scanning mutations. The transposon showed contains genes for a protein fusion reporter gene and an antibiotic resistance (selector). Inserts are selected with the selector and those inserts in the correct orientation and reading frame are identified with the reporter. The bulk of the transposon is excised using a specific cleavage site and ligation leaving the linker sequence in various random locations. See Williams Reznikoff for a modified version of this figure.

cleavage beyond the boundaries of the transposon followed by ligation) as a cloning tool for adjacent DNAs.

d. Origins of DNA transfer.
e. End recognition sites for an alternative transposase, thus constructing a composite transposon (a use for including alternative transposon end recognition sites will be described subsequently).

The Advent of *In Vitro* Transposition Systems

The initial applications of transposon technology were performed with *in vivo* transposition reactions. Typically, the investigator would construct a transposon within a so-called suicide vector. Suicide vectors cannot replicate within the target host under defined conditions. In many cases, the gene encoding the transposase would be located outside of the boundaries of the transposon. The first consequence of the suicide vector design is that the only way in which the transposon-encoded selectable marker could be inherited by the target host would be as a consequence of the transposon transposing off of the suicide vector into the genome of the host. The second consequence would be that the transposase gene would be lost from the host cell. The latter result is important because it would prevent subsequent confounding transposition events.

The above systems were typically tailor-made for work with *E. coli* and *S. typhimurium*. Moreover, they often required genetic manipulations that may not be familiar to today's molecular biologists. In addition, these approaches do not lend themselves to transposition procedures that are targeted to defined DNA regions. These limitations have been addressed as a result of the development of *in vitro* methodologies for studying transposition. Although in most cases, *in vitro* transposition studies were directed at achieving a basic science molecular understanding of DNA transposition mechanisms, the obvious fruit of these studies was the development of molecular genetic tools based on these *in vitro* technologies in whole or in part. The key transposition systems that were developed into *in vitro* molecular genetic tools include Ty1, Tn7, Tn5, Mariner, Mu, and Tn552. For original references and descriptions of these systems see Further Reading.

The most obvious application of the *in vitro* technologies is performing the transposition events on target DNA in the test tube and then introducing the mutagenized DNA into the target cells. The main advantage of this technology is that the target can be restricted to one specific DNA sequence; in some cases an individual gene or gene segment, and in other cases a bacterial artificial chromosome or virus genome. One use of the latter is in the application of transposition technology for high-throughput DNA sequencing (the transposon contains two divergent mobile primer binding sites).

A second important derivative of the *in vitro* studies is the ability to perform a partial *in vitro* reaction (constructing transposase–transposon DNA complexes *in vitro*) followed by electroporating or micro-injecting the transposase–transposon complexes into living cells after which the transposase integrates the transposon DNA into the cells' genomes. This combined *in vitro–in vivo* methodology has only been published for the Tn5 and Mu systems.

The specific applications of both of the above techniques are mentioned in detail below.

Genome-Wide Knockout Analyses

An important approach to genome functional analysis is to generate knockout mutations in as many genes as possible. Obviously, transposons can be the mutagen of choice. The mutagen is highly efficient and in most cases the resulting mutation is an absolute knockout. The mutagen can be delivered in such a way that the great majority of survivors only have single unique mutations, the distribution of mutation sites is relatively random, and the location of individual mutation sites is easy to determine. There are two general approaches that use this methodology. First, one generates a large library of viable insert mutations and then screens or selects for the mutants with the desired phenotype. That is exactly the approach that I used as a new independent investigator when I accidentally isolated a phosphoglucose isomerase-defective mutant. With the advent of genome sequencing information and techniques such as microarray analyses, one can now identify and analyze several different mutants that have similar phenotypes at the same time. For instance, a number of research groups have described techniques in which a large collection of inserts is interrogated in bulk for their members by generating runoff transcripts that include both ends of the transposable element and adjacent DNA and then hybridizing the RNAs to microarrays, thus identifying which inserts are present in the collection (**Figure 3**). By presenting the collection with particular growth challenges and repeating the microarray analyses, one can determine which inserts in which genes cause growth impairment. Thus, this procedure identifies the phenotypes (nutritional requirements) for a class of inserts.

The second approach is designed to identify putative essential genes. In this technology, a large insert library (hopefully a saturated collection with all genes suffering inserts within the collection) is generated. The experimentalist then determines which genes fail to have any inserts represented in the collection. One particular execution of this essential gene hunt was described by Svetlana Gerdes and colleagues in 2002. The pool of transposable element inserts is interrogated by polymerase chain reaction (PCR) analysis using a transposon-based primer and one of the

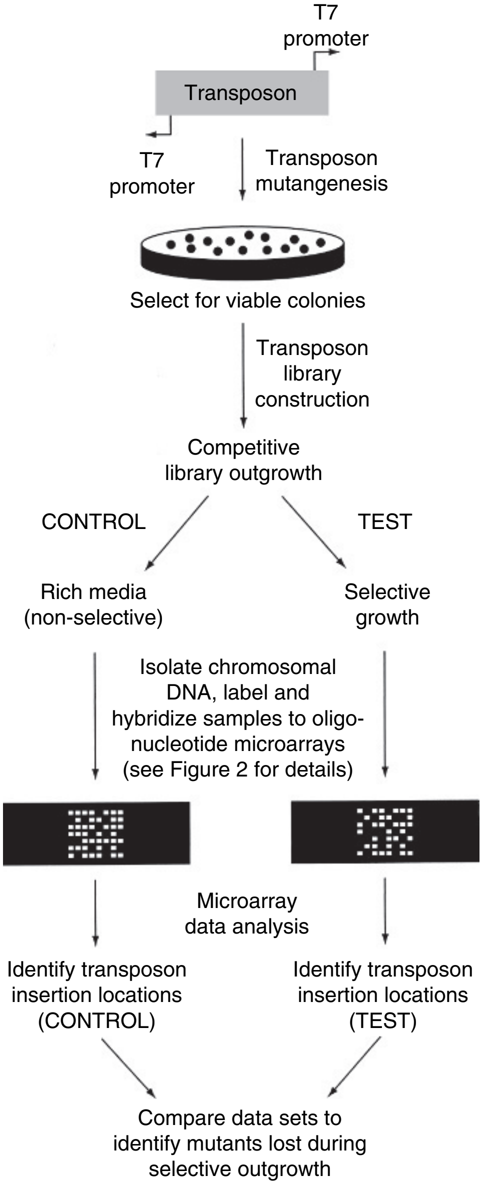

T7
promoter
Transposon
T7
promoter
Transposon
mutangenesis
Select for viable colonies
Transposon
library
construction
Competitive
library outgrowth
CONTROL
TEST
Rich media
(non-selective)
Selective
growth
Isolate chromosomal
DNA, label and
hybridize samples to oligo-
nucleotide microarrays
(see Figure 2 for details)
Microarray
data analysis
Identify transposon
insertion locations
(CONTROL)
Identify transposon
insertion locations
(TEST)
Compare data sets to
identify mutants lost during
selective outgrowth

Figure 3 Genome-wide microarray screening of transposon insertions. The identification, localization, and tracking of multiple transposon inserts can be accomplished by using microarray hybridization. In one version of this technology, the transposon is constructed to have outward-facing T7 promoters. The collection of inserts is harvested, the DNAs extracted, and probes of the DNA sequences adjacent to the inserts are generated using T7 RNA polymerase. The RNA is labeled and hybridized to appropriate microarrays. Reproduced from Winterberg KM and Reznikoff WS (2007) Screening transposon mutant libraries using full-genome oligonucleotide microarrays. In: Kelly T Hughes and Stanley R Maloy (eds.) *Advanced Bacterial Genetics: Use of Transposons and Phage for Genomic Engineering, Methods in Enzymology Series*, Vol. 421, pp. 110–125. San Diego, CA: Elsevier Inc.

several strategically located genome primers. All of the viable inserts are represented by PCR products of predicted sizes, whereas essential genes are tentatively identified by the absence of blocks of PCR products.

The assumption from these studies is that if no inserts for a particular gene are found in the collection, the gene must encode an essential function. However, there are alternative trivial explanations for not finding inserts in a specific gene, such as bad luck or target sequence biases. Therefore, the investigator needs to confirm the identification of a particular gene being essential by other means, such as deletion analysis or individual targeted gene studies that are described below.

Transposable Element-Based Deletion Studies

Transposable elements of the composite transposon class have the capacity to generate adjacent deletions. In this section, I describe the use of this property to study the essentiality of genes (or groups of genes). In a subsequent section, I describe how composite transposon deletion generation can be used to generate nested families of protein deletions.

Composite transposons (such as Tn5 or Tn10) can be thought of as being composed of four different types of transposable elements depending on the precise recognition end sequences that are chosen by the transposase for synaptic complex formation (**Figure 4**). Using the nomenclature presented in **Figure 4**, one can see that Tn5 transposition will involve $O_LER–O_RER$ synapsis. But it is also possible to have $O_LER–I_LER$ or $I_RER–O_RER$ synapsis, in which case one would have IS50L or IS50R transposition, respectively. Of interest to adjacent DNA deletion formation is the possibility of having $I_RER–I_LER$ synapsis, in which case a new transposable element has been formed; the IS50 elements and the donor DNA now compose the transposable element (**Figure 4**).

$I_RER–I_LER$ intramolecular transposition results in one of the two types of DNA rearrangements for the DNA between these two ends, either a deletion or an inversion (**Figure 5**). The $I_RER–I_LER$ intramolecular deletion formation potential has been developed into a practical chromosome deletion tool by utilizing a transposase that is selective for I-ER sequences (as apposed to O-ER sequences) and by genetically marking the components of the new composite transposon so that only deletions are isolated. The resulting system has been used to delete random sections of the *E. coli* chromosome and thereby can be used to define which genes are not essential (they can be deleted and still yield a viable organism).

Targeting Individual Genes

The most important consequence resulting from the development of *in vitro* transposition systems is the ability

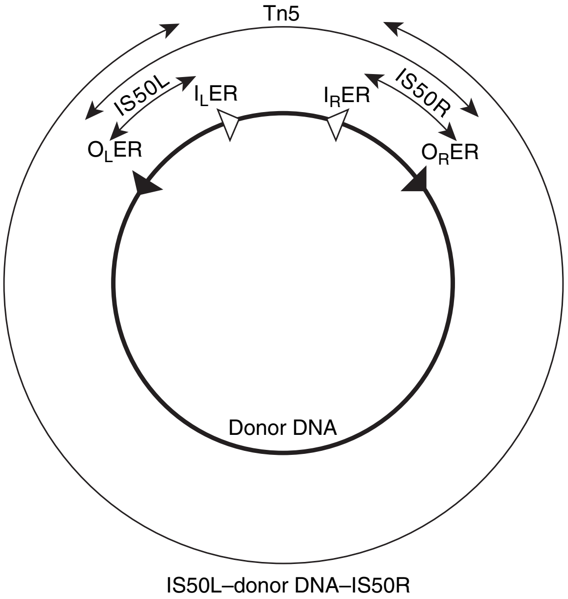


Figure 4 Composite transposons can give rise to four types of transposons. Composite transposons such as Tn5 contain two identical or nearly identical insertion sequences (IS50L and IS50R in the case of Tn5) that bracket additional genes. Depending on which end recognition (ER) sequences are chosen by the transposase during synapsis, four different transposons can be mobilized: Tn5 mobilization involves O$_L$ER–O$_R$ER synapsis, IS50L mobilization involves O$_L$ER–I$_L$ER synapsis, IS50R mobilization involves I$_R$ER–O$_R$ER synapsis, and IS50L–donor DNA–IS50R mobilization involves I$_L$ER–I$_R$ER synapsis.

to target transposition to specified DNA sequences such as individual genes. From this general technology a number of specific applications are derived. An obvious use of targeted *in vitro* transposition is to generate gene-specific knockouts and then attempt to introduce the knockouts as substitutions for the intact gene in the host organism. If the knockout organism can be isolated and propagated, the disrupted gene is not essential. If the experimenter is unable to isolate cells that contain the insertion, the negative result is *prima facie* evidence that the gene is essential. As described below, other applications of targeted *in vitro* transposition lend themselves to a variety of techniques that allow the analysis of protein structure-function.

Protein Structure-Function Studies: Generating Random Nested Deletions

As mentioned above, intramolecular transposition can be used to generate deletions. A straightforward adaptation of the intramolecular transposition/deletion technology to generate nested deletions in a protein-encoding gene first requires the construction of a transposon containing the target gene through recombinant DNA techniques. Once it is constructed, intramolecular transposition will generate a nested family of deletions of the target gene. These deletions can be used to map epitopes or specific domains of interest to the investigator.

Protein Structure-Function Studies: Generating In-Frame Microinsertions, Deletions, and Substitutions

Transposons have become widely used (and commercially available) tools for generating in-frame linker insertions. The principle, as outlined in **Figure 2**, typically follows the general steps mentioned below. At first, one uses translational fusion technology (inserting a reporter gene lacking transcription and translation initiation signals into the target gene of choice) to capture inserts with the correct orientation and reading frame. Then one excises the bulk of the transposable element either using rare restriction enzyme digestion followed by ligation or a site-specific recombination system leaving an in-frame insertion, whose sequence is dictated by the residual transposon sequence. Of course, a precisely constructed transposon needs to be used for this technology. The small inserts can be used to map structural domains of the protein (functional proteins typically result only from insertions in unstructured regions) or to insert an epitope or protease target sequence.

An exciting extension of this insertion technology has been developed in the laboratory of Dafydd Jones. In this technique, a specialized version of a mini-Mu transposon is used for the initial mutagenesis, and the off-set cleavage activity of a type IIS restriction enzyme is used to perform the excision of the bulk of the transposon. Depending on precisely how the procedure is applied, and whether an intermediate cloning is performed or not, the technique can be utilized to generate precise 3-bp (base pairs) deletions or additions, or precise 3-bp substitutions at the site of transposon insertion. The latter is particularly powerful. Imagine generating a random collection of single insertions on the target gene of choice and then generating known sequence substitutions at each of the sites.

Protein Structure-Function Studies: Generating Random Protein Fusions

In all the above protocols, a constant sequence (the transposon) is juxtaposed against a random sequence (the target). By utilizing a specifically designed composite transposon and transposases that were specific for the two types of recognition end sequences (I-ER and O-ER), we developed techniques that fused two genes in a random fashion with a sequence composed of an I-ER and O-ER between the two fused gene sequences (**Figure 6**). We applied this technology only once. The results were that active product fusion proteins were generated only when the resulting fusion partners were in the

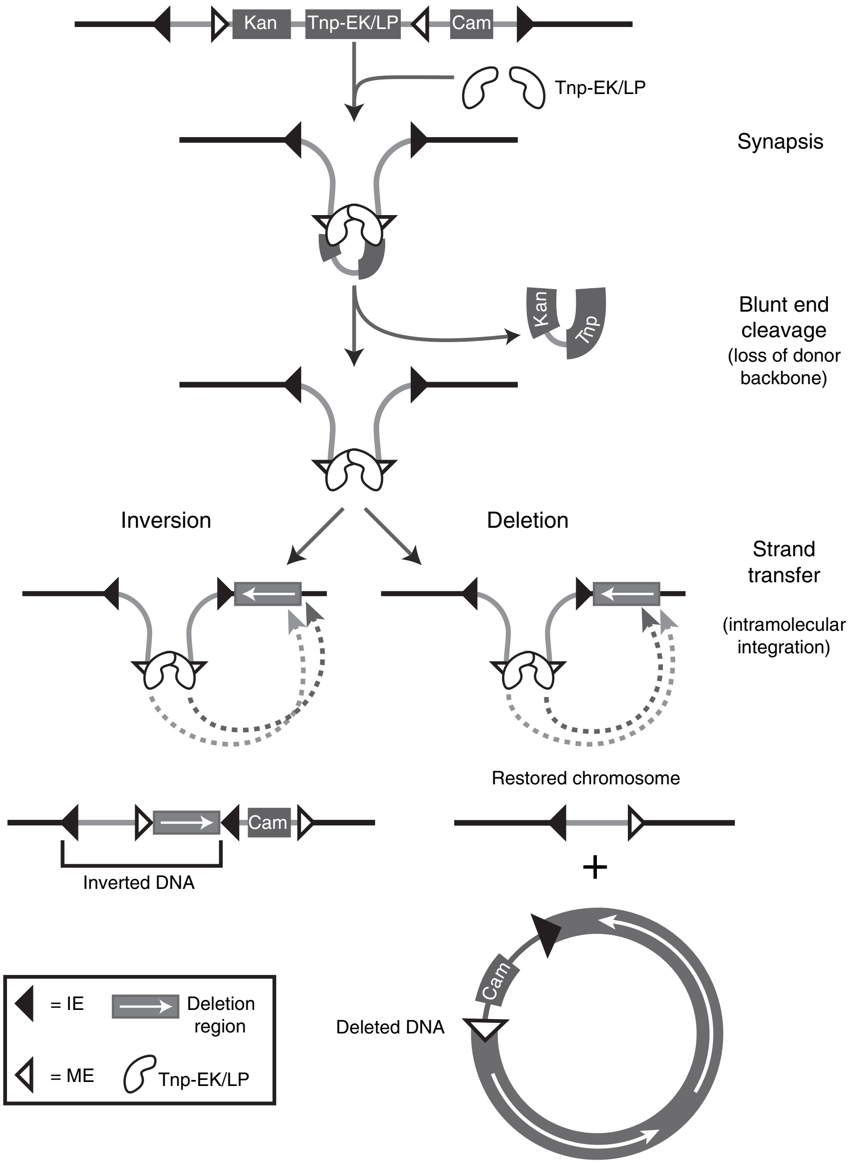
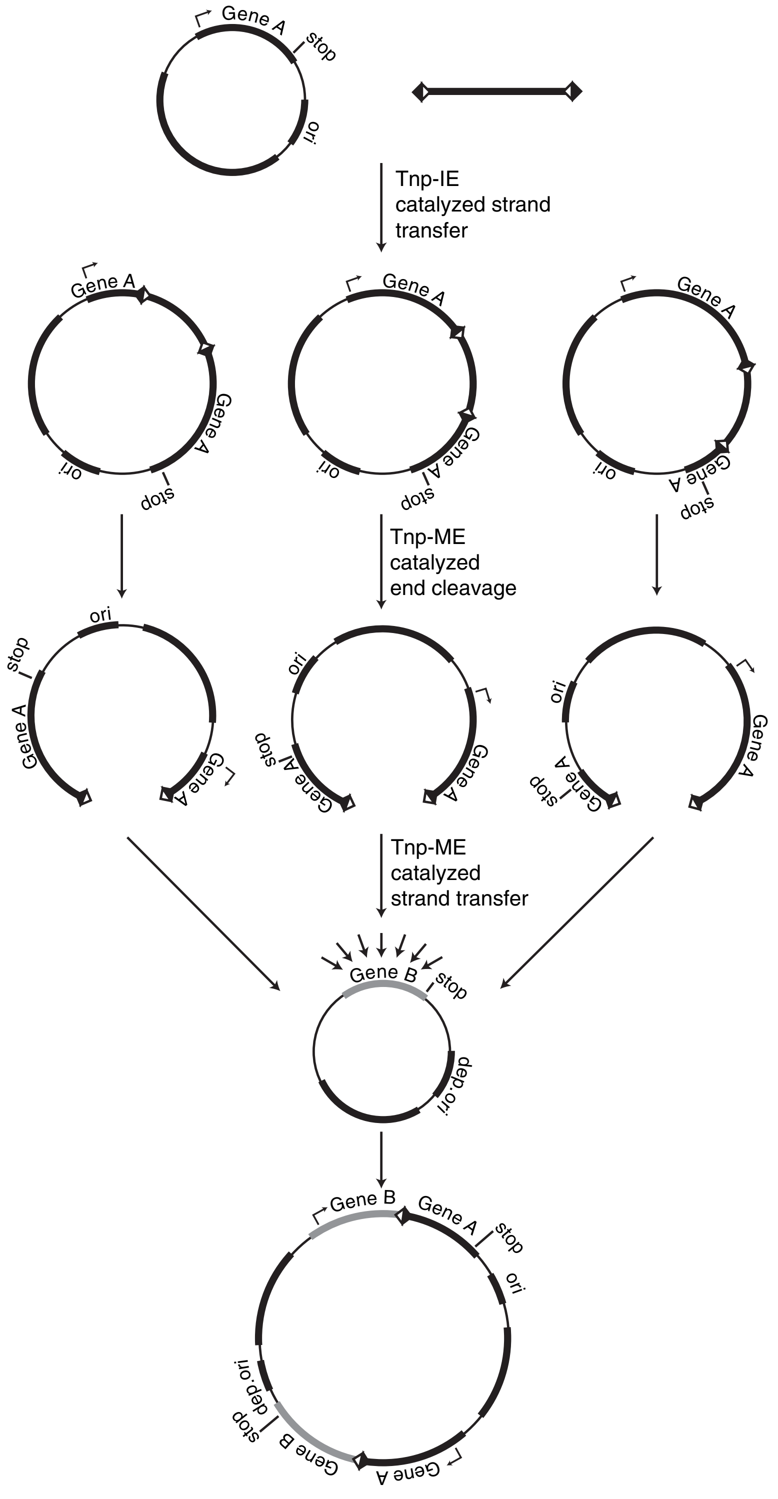

Kan
Tnp-EK/LP
Cam
Tnp-EK/LP
Synapsis
Kan
Tnp
Blunt end cleavage
(loss of donor backbone)
Inversion
Deletion
Strand transfer
(intramolecular integration)
Restored chromosome
Cam
Inverted DNA
+
Cam
Deleted DNA
= IE
Deletion region
= ME
Tnp-EK/LP

Figure 5 Intramolecular transposition and adjacent gene deletion formation. Intramolecular transposition that utilizes I_LER–I_RER synapsis as shown in **Figure 4** has been used to generate adjacent chromosomal deletions as a means to define nonessential genes and to reduce the size of the *Escherichia coli* chromosome. A transposase specific for the open triangle end recognition sequences forms synaptic complexes, cleaves the DNA free of the Kan-Tnp-EK/LP-encoding donor DNA, and catalyzes intramolecular transposition, which can generate deletion formation to give the ‘restored chromosome’ shown. Reproduced from Goryshin IY, Naumann TA, Apodaca J, and Reznikoff WS (2003) Chromosomal deletion formation system based on Tn5 double transposition: Use for making minimal genomes and essential gene analysis. *Genome Research* 13: 644–653.

same reading frame and orientation and when the proteins were fused utilizing unstructured regions between secondary structure domains. There is no obvious reason why this technology cannot be applied in generating a variety of functional fusion proteins.

Protein Structure-Function Studies: Generating Random Reporter Gene Fusions

The ability to generate reporter fusions to a target protein is very useful. An example is suggested above in the ‘Landmarks’ in which a *Lac*I-CFP (cyan fluorescent protein) and TetR-YFP (yellow fluorescent protein) were used to locate Mariner constructs that contained $(lacO)_n$ or $(tetO)_n$, respectively. Typically, tagged fusion proteins are constructed using recombinant DNA technology to generate N- or C-terminal fusions. This construction method makes the assumption that one or the other type of fusion will maintain optimal protein function and probably eliminates the possibility of using the reporter as a probe for changes in protein conformation.


Gene A
stop
ori
Tnp-IE
catalyzed strand
transfer
Gene A
Gene A
ori
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Tnp-ME
catalyzed
end cleavage
ori
stop
Gene A
Gene A
Tnp-ME
catalyzed
strand transfer
Gene B
stop
dep.ori
Gene B
Gene A
stop
ori
dep.ori
stop
Gene B
Gene A

Figure 6 Two sequential transposition events used to generate random gene fusions. The gene fusion technology utilizes a transposase that recognizes closed triangle end recognition sequences to first insert the transposon into target gene A and then a transposase that recognizes open triangle end recognition sequences to insert the newly formed transposon carrying the two halves of gene A into gene B. Some of the resulting products encode random fusions of genes A and B. Reproduced from Naumann TA, Goryshin IY, and Reznikoff WS (2002) Production of combinatorial libraries of fused genes by sequential transposition reactions. *Nucleic Acids Research* 30: e119, with permission from Oxford University Press. See also Williams Reznikoff.

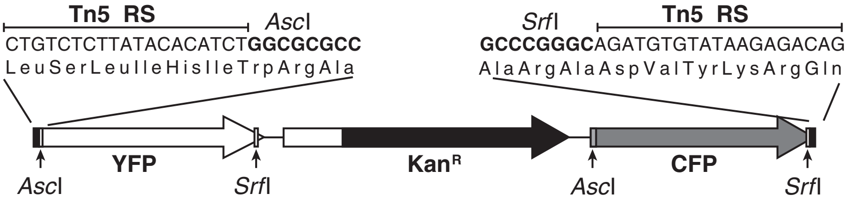
Tn5 RS
AscI
CTGTCTCTTATACACATCTGGCGCGCC
LeuSerLeuIleHisIleTrpArgAla
SrfI
Tn5 RS
GCCCGGGCAGATGTGTATAAGAGACAG
AlaArgAlaAspValTyrLysArgGln
YFP
KanR
CFP
AscI
SrfI
AscI
SrfI

Figure 7 Transposon for generating random yellow fluorescent protein (YFP) (or cyan fluorescent protein, CFP) fusions. The transposon shown will generate Kan^R, YFP fusion as a result of insertions in the correct orientation and reading frame into a target gene. Digestion with *Srf*I and ligation will excise the bulk of the transposon, generating a fusion of both the N- and C-terminal portions of the target gene sequence to YFP. Alternatively, digestion with *Asc*I followed by ligation will generate a fusion to CFP. Reproduced from Reznikoff WS (2006) Tn5 transposition: a molecular tool for studying protein structure-function. *Biochemical Society Transactions* 34(part 2): 320–323 and Sheridan DL and Hughes TE (2004) A faster way to make GFP-based biosensors: Two new transposons for creating multicolored libraries of fluorescent fusion proteins. *BMC Biotechnology* 4: 17–25.

One can also make CFP or YFP fusions using transposon technology. By using the construct described in **Figure 7**, one can search a wide variety of fusions for the ones that have optimal properties. The concept is to initially generate random YFP$^+$ fusions (in the correct orientation and in frame) to the gene of interest. Following the fusion generation, an in-frame portion of the transposon (including the translation termination signal) is removed using *Srf*I cleavage and ligation. The resulting product encodes the N-terminal target-YFP (active)-C-terminal target. These fusion constructs are then examined to find those that have maintained maximal target protein activity and/or those that make YFP emission sensitive to target protein environment or function. The construct is designed also to allow the generation of CFP fusions from the same inserts.

Conclusion

Transposons are powerful tools in the whole genome structure studies and in the analysis of protein (and RNA) structure-function. Although there is an energy barrier to their adoption in some laboratories, once accepted they combine ease of use, great flexibility, and straightforward interdigitation with other technologies.

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Tuberculosis: Molecular Basis of Pathogenesis

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Defining Statement
Overview
TB Pathogenesis
Bacterial Factors Involved in TB Pathogenesis
TB Latency
Drug Resistance and Pathogenesis
Conclusions
Further Reading

Glossary

DAT and PAT Diacyltrehaloses and polyacyltrehaloses.
dormancy *Mycobacterium tuberculosis* during latency.
LAM Lipoarabinomannan.
latency Tuberculosis infection with no signs of active disease.
ManLAM Mannose-capped LAM.
MDR-TB Resistance to isoniazid and rifampin.
PDIM/DIM Phthiocerol-dimycocerosate.
PGL Phenolic glycolipid.
SL Sulfolipid I; trehalose sulfated at the 2′ position and esterified with palmitic acid and multimethyl-branched fatty acids.
TB–HIV Coinfection with *M. tuberculosis* and HIV.
tuberculosis Highly infectious disease caused by *Mycobacterium tuberculosis* (tubercle bacillus; first described by Robert Koch, 24 March 1882) in evidence since 4000–2000 BC.
XDR-TB MDR plus resistance to any fluoroquinolone and at least one of the injectable drugs (capreomycin, kanomycin, amikacin).

Abbreviations

DAT	2,3-di-*O*-acyltrehalose
DosR	dormancy survival regulator
LAM	Lipoarabinomannan
LM	Lipomannan
MPM	mannosyl-β-1-phosphomycoketides
MR	mannose receptor
PAT	polyacyltrehalose
PDIM	phthiocerol dimycocerosate
PGL	phenolic glycolipid
***p*-HBADs**	*p*-hydroxybenzoic acid derivatives
TB	tuberculosis
TDM	trehalose 6,6′-dimycolate

Defining Statement

The lipids of *Mycobacterium tuberculosis*, long regarded as chemical oddities, are now centerpiece in our understanding of the characteristic pathogenesis of tuberculosis, in its latent, and highly infections states.

Overview

With the greater awareness of the public health dimensions of the tuberculosis (TB) problem, there are many outstanding recent reviews, books, monographs, and so on, on the subject. The preferred habitat of *Mycobacterium tuberculosis* is the human lung, although extrapulmonary TB, of a wide range of conditions and diverse pathology, is serious and can also be fatal. The dimensions of present-day TB in all its manifestations, such as drug-sensitive forms, multiple and extensive drug-resistant forms (MDR/XDR-TB), TB–HIV coinfection, and latent TB, are well documented. TB is the leading cause of death from a single bacterial infection and a leading lethal opportunistic infection in HIV-infected individuals. Based on tuberculin/PPD positivity, it is estimated that one-third of the world's population is infected with *M. tuberculosis*, the vast majority immunologically capable of containing the infection. Still, there may be 14 million active cases worldwide, many sputum-positive and therefore highly infectious, with over eight million new cases per annum and up to 1.6 million deaths. MDR and XDR strains of *M. tuberculosis* are found worldwide; it is estimated that there are about 500 000 such cases. The Centers for Disease Control and Prevention reported that between 2000 and 2004, 20% of all reported TB cases were MDR and 2% were XDR-TB. In a recent study conducted in a rural area of South Africa, of 475

TB patients, 39% were infected with MDR-TB strains and 6% were XDR-infected. Of the 53 patients with XDR-TB, 52 died during the study; the average number of days from diagnosis to death for those in the population (the majority) coinfected with HIV and XDR strains was 16.

TB Pathogenesis

Within the human lung, the maximum microbial population density is in the inner zone of the wall of a chronic open cavity, an environment most suited for the noted lifelong persistence of *M. tuberculosis*. Dissemination of *M. tuberculosis* occurs from the lung. Of the many infected, less than 10% develop the disease; the majority harbor latent TB, but when it occurs, TB transmission, by droplet nuclei, mostly through coughing, is the primary route, and the only epidemiologically important source of the disease.

When bacteria in general reach the human lung, they are usually engulfed and destroyed by the bronchial alveolar macrophages. In the case of *M. tuberculosis*, it is also internalized by alveolar macrophages, which are triggered to cross the epithelial layer of the lung. The subsequent cytokine-mediated inflammatory response leads to the recruitment of more macrophages from the circulation, providing further host cells for the replicating bacteria, but also for the formation of granuloma, again induced by immune responses to *M. tuberculosis* components. Normally, the immune response is protective and the infection remains in a chronic, latent, or containment state marked by a low-level stable bacterial load. Progression to active disease, stimulated by a variety of risk factors, notably T-cell depletion, is marked by tissue destruction, necrosis, release of extracellular bacteria into the lung, and subsequently communal transmission.

M. tuberculosis has developed mechanisms to survive the intracellular onslaught experienced by most other bacteria. Detection of *M. tuberculosis* on the macrophage surface involves complex interactions of bacterial ligands and host cell receptors. During phagocytosis, ManLAM attaches to the Fc receptor, mannose receptor (MR), and/or DC-SIGN. Detection of these complexes by the pathogen recognition receptor, TLR-2, induces a signaling cascade mediated by the TLR-2 adaptor protein TIRAP. Total activation of these primed macrophages is effected by soluble immune modulators, notably IFNγ, secreted by T-lymphocytes. Fully activated macrophages, however, fail to eradicate *M. tuberculosis* but do restrict its growth. *M. tuberculosis*-containing phagosomes within the macrophage fail to fuse with lysosomal compartments, since they retain many of the characteristics of the early endosome. The particular features of the *M. tuberculosis* phagosome, and the molecular basis of failure to mature, have been well documented. The result is failure to fuse with the lysosomal compartment of the cell and with it the effects of the killing mechanisms associated with the lysosomal range of hydrolytic enzymes, ROIs, RNIs, and of proinflammatory cytokine (e.g., TNFα) are ameliorated. This is the essence of *M. tuberculosis* pathogenesis and disease induction.

Bacterial Factors Involved in TB Pathogenesis

Some of the characteristic components of the cell wall of *M. tuberculosis* have been those most implicated in all aspects of TB pathogenesis. The structure and architecture of the cell wall of *M. tuberculosis* has been reviewed extensively. It consists of a core structure composed of peptidoglycan covalently attached via a linker unit to a linear galactofuran, in turn attached to several strands of highly branched arabinofuran, in turn attached to mycolic acids. The mycolic acids are oriented perpendicular to the plane of the membrane and provide a lipid environment responsible for much of the pathogenesis of TB. Intercalated within this lipid environment are the phthiocerol dimycocerosate (PDIM), cord factor/dimycolytrehalose, other acyltreholases, the sulfolipids, the phosphatidylinositol mannosides, and the related lipomannan (LM) and lipoarabinomannan (LAM), and others. Knowledge of their roles in signaling events, in pathogenesis, and in the immune response such as activation of CD1-restricted T-cells by mycolic acids, the recognition that antigen 85, one of the most powerful protective antigens of *M. tuberculosis*, is a mycolyltransferase, and that LAM, when 'capped' with short mannose oligosaccharides, is involved in phagocytosis of *M. tuberculosis*, is now emerging.

The PI-Containing Ligands

The structures of the more important bacterial ligands involved in TB pathogenesis are shown in **Figures 1** and **2**. ManLAM, that is, LAM with the mannose-containing caps, and its precursors, LM and the PIMs, also found in appreciable quantities in *M. tuberculosis*, are the most important bacterial ligands in infection and pathogenesis. ManLAM and some of the PIMs, during phagocytosis, bind to the MR and DC-SIGN, and Schlesinger and group have speculated that MR binding initiates inhibition of phagosome maturation and subsequent intracellular survival of the bacteria, whereas the DC-SIGN pathway will result in phagolysosome infusion and bacterial clearance. In addition, ManLAM has been implicated in myriad other aspects of the infection process, such as regulation of proinflammatory cytokines such as IL-6, TNFα, and the cytocidal oxidative burst; involvement in the inhibition of phagosome–lysosome fusion; escape from the phagosome in intracellular vesicles to the intracellular trafficking network within the

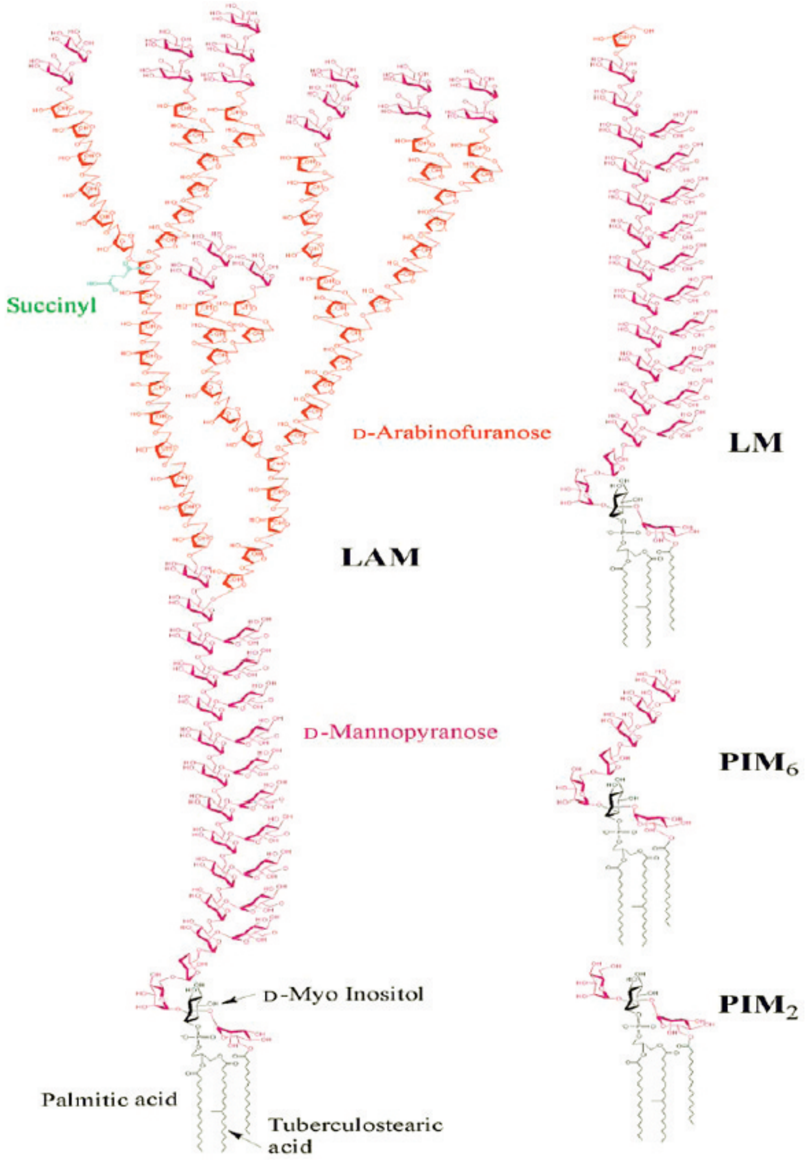

Figure 1 Proposed structures of ManLAM, LM, and some of the PIMs of *M. tuberculosis* based on current information. Reproduced from Scherman H. Ph.D. Thesis, Colorado State University.

macrophage; involvement in antigen processing and presentation to T-cells via the CD-1 pathway; and inhibition of *M. tuberculosis* induced-apoptosis through alteration of Ca^{2+}-dependant signaling. The intriguing concept that the structural arrangement of the Man-caps of ManLAM equates to those on some eukaryote glycoproteins with a preponderance of 2-α-Manp residues has been advanced and the outcome is survival through the MR pathway. Interestingly, LAM and some of the PIMs (**Figure 1**) have been shown to bear similar but at times distinctly different but complementary roles to ManLAM in the infection process, such as participation in phagocytosis through CR3 and, perhaps, facilitating fusion with early endosomal compartments. There is also evidence that LM specifically associates with DC-SIGN rather than MR.

However, it should be borne in mind that most of these studies were conducted with the purified mycobacterial products delivered as liposomes or on coated beads. Some of these effects will have to be reevaluated once isogenic mutants devoid of LAM, LM, and so on, are available. Hence, recent efforts to define the biosynthesis of the PI-containing ligands and generate LAM-defective mutants are important.

The Polyketide-Derived Lipids

The roles of the polyketide-derived lipids of *M. tuberculosis* (specifically those containing phthiocerol and trehalose) in the pathogenicity of the organism are at a more advanced level in that their genetic and biosynthetic

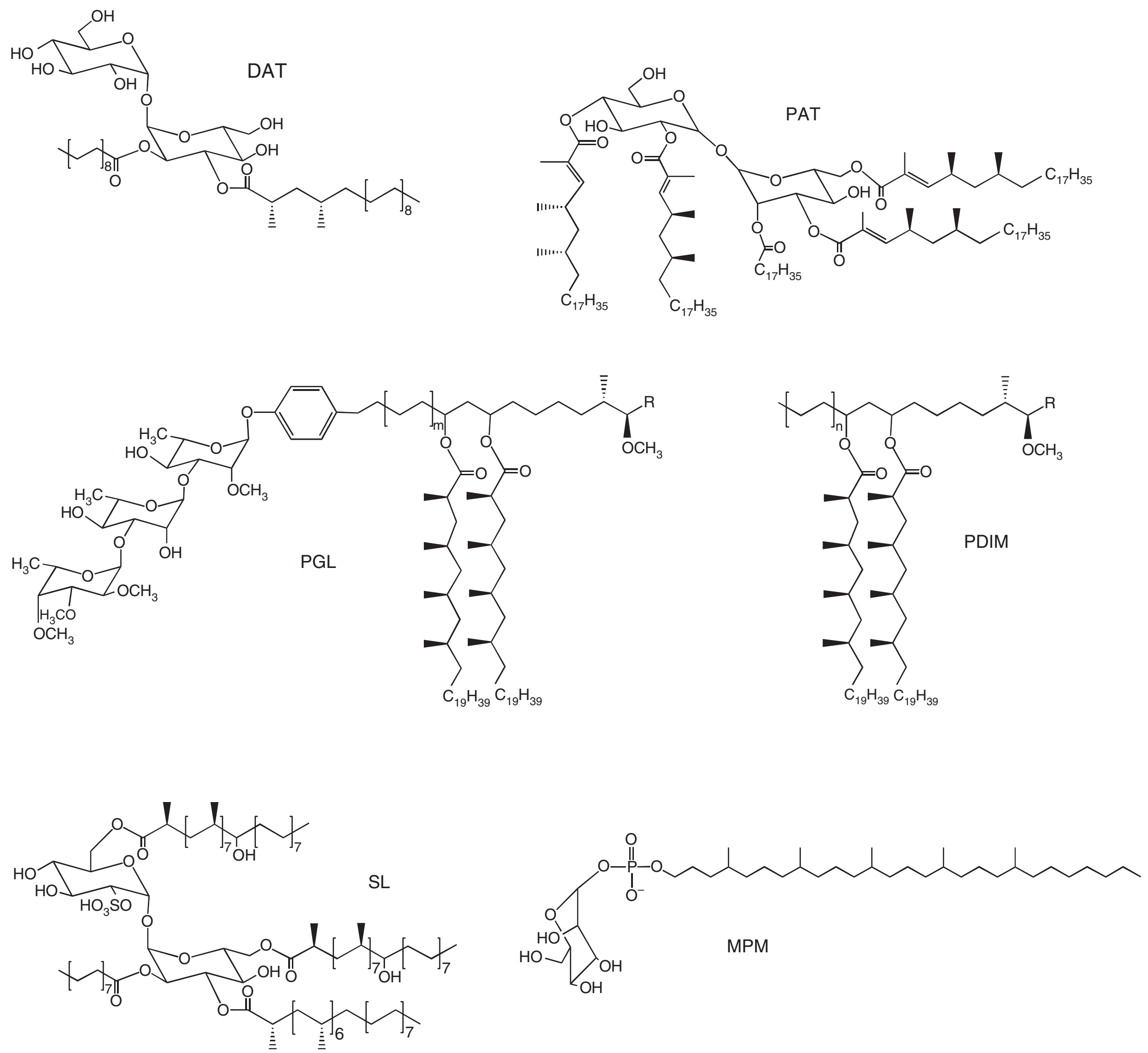

Figure 2 Structures of some of the polyketide-containing lipids of *M. tuberculosis* involved in various aspects of infection and pathogenesis. Reproduced from Jackson M, Stadthagen G, and Gicquel B (2007) Long-chain multiple methyl-branched fatty acid-containing lipids of *Mycobacterium tuberculosis*: Biosynthesis transport, regulation and biological activities. *Tuberculosis* 87: 78–86. In DAT (2,3-di-*O*-acyltrehalose), trehalose is esterified with stearic acid and the multimethyl-branched mycosanoic acid. In PAT (polyacyltrehalose), trehalose is esterified with stearic acid and the multimethylbranched mycolipenic acids. In PDIM, the long-chain β-diol (phthiocerol moiety) is esterified with two mycocerosic acids; $n = 10–11$; $R = –CH_2–CH_3$ or $–CH_3$. The lipid core of PGL consists of phenolphthiocerol esterified by mycocerosic acids; $m = 7–8$; $R = –CH_2–CH_3$ or $–CH_3$. The trisaccharide substituent consists of 2,3,4-tri-*O*-methyl-α-L-Fuc*p*-(1→3)-α–L-Rha*p*-(1→3)-2-*O*-methyl-α-L-Rha*p*. The major sulfolipid, SL-I (2,3,6,6′-tetraacyl α-α′-trehalose-2′-sulfate), is shown. In SL-I, trehalose is sulfated at the 2′ position and esterified with palmitic acid and the multimethyl-branched phthioceranic and hydroxyphthioceranic acids. The predominant mannosyl-β-1-phosphomycoketides (MPM) from *M. tuberculosis* H37Rv consists of a mannosyl-β-1-phosphopolyketide with a C32 4,8,12,16,20-pentamethylpentacosyl chain.

origins have been well defined and, consequently, isogenic mutants have emerged to allow meaningful functional studies.

The *M. tuberculosis* genome sequence contains 27 putative *pks* genes clustered into about 11 loci, and the function of these has mostly been defined in the context of the biosynthesis of the phthiocerol and branched fatty acids of these key ligands (**Figure 2**). Consequently, various mutants have been generated, mostly through signature-tagged transposon mutagenesis. Historically, cord factor/trehalose 6,6′-dimycolate (TDM) is the most studied; the peculiar and characteristic toxicity in mice whereby a few repeated intraperitoneal injections of small amounts dissolved in paraffin oil kills a majority of the animals, apparently through a physical attack on mitochondria membranes, and failure of oxidative phosphorylation, have also been extensively studied. Incidentally, TDM can have powerful immunogenic, granulomagenic, adjuvant, and antitumor activity.

The polyketide-derived lipids whether of the phthiocerol- or trehalose-containing variety have been implicated in all aspects of *M. tuberculosis* pathogenicity: early interaction with macrophages, dendritic, and epithelial cells; the intracellular fate of *M. tuberculosis*; its multiplication and persistence within the infected host; and modulation of the host immune response. Of these, the effects of DIM/PDIM and the phenolic glycolipids (PGLs) of *M. tuberculosis* and their precursor metabolites (**Figure 3**) are the most studied. The synthesis of the PGLs and DIMs and their translocation to the surface of *M. tuberculosis* require a genomic locus containing 5 *pks* genes (*pps* A–E) responsible for phthiocerol synthesis and at least 16 other genes involved in initial fatty acid priming synthesis, mycocerosic acid synthesis, condensation of mycocerosic acids and the phthiocerols, and translocation. Other *pks* genes in conjunction with those responsible for methylation and glycosylation ensure the synthesis of PGL, but only in some strains of *M. tuberculosis*. Insertion of transposons into several of these genes with consequent deletion of the DIM end product, or, alternatively, synthesis but failure to be fully transported allowed cellular and animal functional studies, indicating that DIM is a virulence factor facilitating the intracellular and extracellular growth of *M. tuberculosis*. These results confirmed the observations of M. Goren in the 1970s with spontaneous DIM-deficient clinical isolates of *M. tuberculosis*. The data clearly implicated DIM in the protection against *M. tuberculosis* through the cytocidal activity of R01s and RNIs produced by activated macrophages and the downregulation of inflammatory cytokines such as TNFα and IL-6.

The production of PGL (**Figure 3**) in certain strains of *M. tuberculosis*, including many of the W–Beijing family,

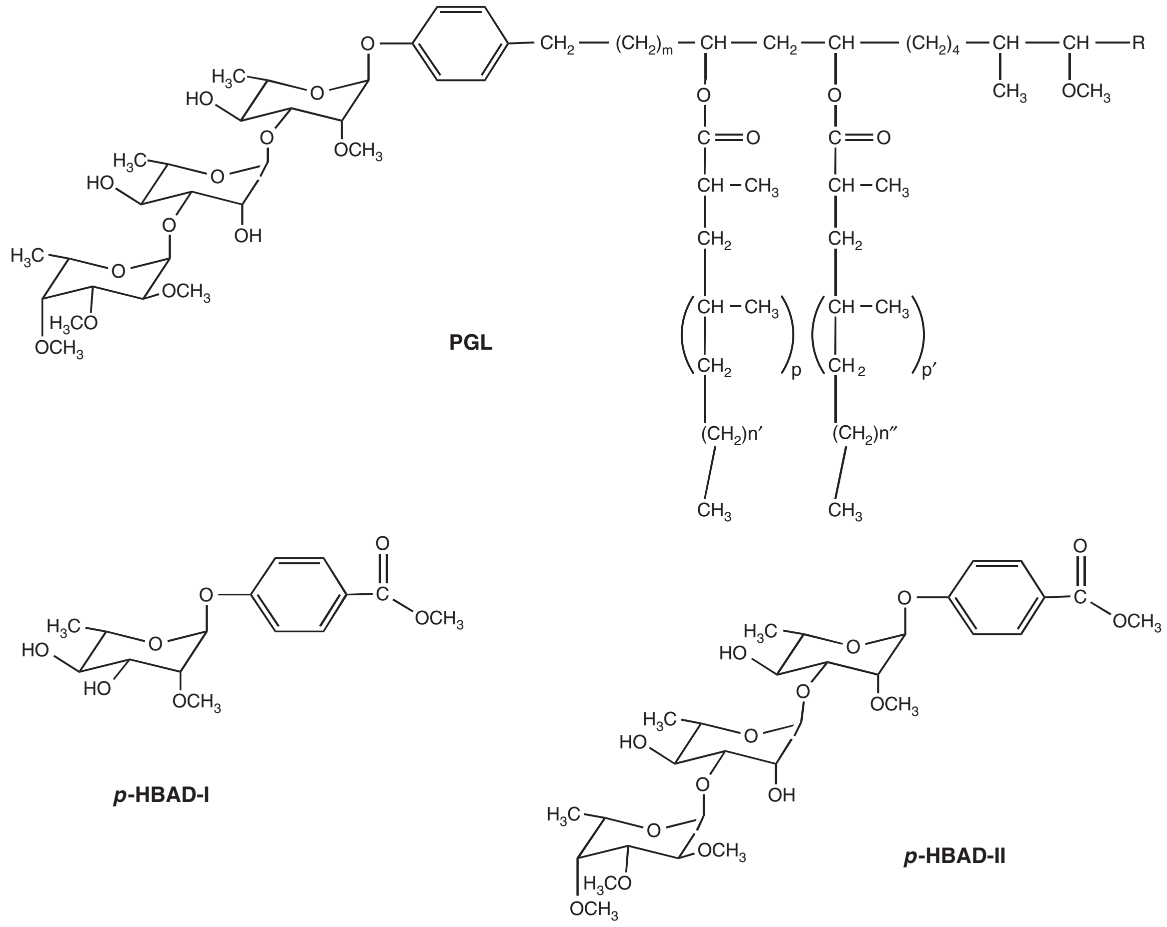

Figure 3 Structures of the PGLtb of *M. tuberculosis* (see **Figure 1**) found only in some strains, including those with a hypervirulent phenotype, among the members of the W–Beijing family. PGLtb is not present in most strains of *M. tuberculosis* including the much used H37Rv strain, due to a frameshift mutation. Instead, these contain the *p*-hydroxybenzoic acid derivatives (*p*-HBAD*)*. The sugars in the *p*-HBADs are those in mature PGLtb (**Figure 1**). Reproduced from Stadthagen G, Jackson M, Charles P, *et al*. (2006) Comparative investigation of the pathogenicity of three *Mycobacterium tuberculosis* mutants defective in the synthesis of *p*-hydroxybenzoic acid derivatives. *Microbes and Infection* 8: 2245–2253.

has been associated with a hypervirulence phenotype in mice, such as the attenuation of the ability to kill mice, and inhibition of the evocation of pro-inflammatory cytokines such as TNFα, IL-12, IL-6, and the monocyte chemotactic protein-I. Interestingly, most isolates of *M. tuberculosis*, including the common H37Rv strain, are devoid of the PGLs due to an inherent frameshift mutation. However, they do produce a set of precursors such as *p*-HBAD (*p*-hydroxybenzoic acid derivative-I and -II and these induce their own spectrum of virulence phenotypes.

TB Latency

The vast population of latent, asymptomatic TB (~1.8 billion) undoubtedly help sustain the TB pandemic; it is estimated that nearly nine million latently infected individuals develop active TB per year, and each of these infect 10–15 others before succumbing to the disease or responding to therapy. The status of the bacterium during this hibernation period, how it evades an effective immune response, how it withstands prolonged chemotherapy, and how it responds to the signals and mechanism of reactivation have not been answered satisfactorily. Most of what we do know arises from models of latency, notably the hypoxic *in vitro* model and the Cornell *in vivo* model. The dormancy survival regulator (DosR) is clearly key to the induction of hypoxic genes and survival, at least in the *in vitro* Wayne model. There is now some evidence that during latency *M. tuberculosis* may be extra-granuloma and in a nonreplicating state and that the introduction of oxygen through whatever means may act as the resuscitation/reactivation trigger. An intriguing aspect of drug resistance and *M. tuberculosis* pathogenesis has recently emerged, namely, the effect that drug resistance determinants can have on bacterial fitness (i.e., virulence or pathogenesis) and the microevolutionary mechanisms of how *M. tuberculosis* adapt to these effects. The phenomenon is of public health interest in light of reports of the enhanced transmissibility of strains such as the W–Beijing family of MDR-TB strains, apparently responsible for more severe forms of the disease.

Drug Resistance and Pathogenesis

Of the several common mechanisms by which bacteria can become resistant to antibiotics, target modification and inactivation of drug-activating enzymes are the most frequent in *M. tuberculosis*; efflux pumps or drug-inactivating mechanisms have not been demonstrated in clinical TB drug resistance. Resistance of *M. tuberculosis* to all known drugs is due to mutations in chromosomal genes; for instance, MDR-TB arises from sequential accumulation of mutations in different genes involved in individual instances of drug resistance due to poor adherence or inappropriate treatment.

Resistance to frontline TB drugs is generally not associated with effects on virulence. However, there are two marked contrasts. It has been known for years that INH resistance can result in loss of both catalase activity and virulence in guinea pigs, and there is good correlation between the two: INH-resistant mutations in the katG gene resulting in loss of enzyme activity also lose virulence, whereas those that retain activity also retain the virulence phenotype.

The contrasting situation has recently been revealed in the case of members of the W–Beijing family of *M. tuberculosis* strains. The W–Beijing family are predominately associated with the MDR phenotype and seemingly are remarkably robust and fit in terms of disease transmission and the severity of the disease. As indicated above, members of the W–Beijing hypervirulent family commonly produce the PGL virulence glycolipid. However, there must be other microbial factors in play, such as mutations in the mutator genes, responsible for greater mutation frequency and better *in vivo* adaptability.

Recent research has related the phenomenon of drug tolerance (drug resistance due to changes in the physiology of the bacterium) and association with the nonreplicating, or dormant/persistent state, the cause of latent TB. The belief is that the dormant organism is in an O_2-deprived and C-starved environment and this is known to be relatively resistant to most anti-TB drugs. The clinical challenge nowadays is the eradication of both replicating and nonreplicating *M. tuberculosis*. The phenomenon of nonreplicating resistors and efforts to avoid their resuscitation and consequent clinical relapse are probably the basis of the prolonged nature of modern-day TB chemotherapy.

Conclusions

Recent research has resulted in spectacular progress in defining the genetic and biosynthetic origins of the myriad and structurally complex virulence factors of *M. tuberculosis* and their roles in the intracellular life and death of the organism. These developments have been complemented by impressive progress in defining protective immunity and immunopathogenesis. However, new challenges have come to the fore: the nature of the dormant bacterium; the immunological basis of dormancy and resuscitation; and chemotherapy of latent TB. The modern phenomena of TB–HIV coinfection, MDR-TB, and XDR-TB have also revealed the phenomenon of hypervirulent, widespread, superfit strains of *M. tuberculosis* in our midst.

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Vaccines, Viral

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General Principles
Live Virus Vaccines
Noninfectious Vaccines
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Glossary

adjaptive immunity B- and T-lymphocyte-mediated memory immune responses that control viral infections through specific interactions with virus-infected cells and virions.

adjuvant Immunologic agent that stimulates the immune system and increases the response to a vaccine without having specific antigenic effect by itself.

attenuation Genetic alteration of infectious viruses to reduce their potential to cause disease.

immunogenicity Capacity to elicit adaptive immunity to proteins of a virus.

major histocompatibility complex (MHC) Cell surface proteins that allow recognition of foreign antigens by the immune system.

protective efficacy Capacity to protect against disease usually caused by a virus.

recombinant DNA The result of combining DNA fragments from different sources.

tropism Pattern of infectivity for cells and organs that is characteristic of a viral pathogen.

Abbreviations

CTL	cytotoxic T lymphocytes
HA	hemagglutinin
HIV	human immunodeficiency virus
HPV	human papillomavirus
IFNα	interferon-α
IFNγ	interferon-γ
IL2	interleukin 2
LAIV	live attenuated influenza vaccine
MHC	major histocompatibility complex
MMR	measles–mumps–rubella
MMRV	measles, mumps, rubella, and varicella
NA	neuraminidase

Defining Statement

The fundamental objective of vaccination against viral pathogens is to induce adaptive immunity in the naive host, which protects from disease upon subsequent exposures to the infectious agent. Adaptive immunity is achieved by inoculation of the host with attenuated infectious virus or with viral proteins administered in a noninfectious formulation.

General Principles

In the case of natural viral infections, a naive host who has never encountered a particular viral pathogen will respond with both innate and adaptive immunity. Innate immune responses are generally initial steps to control and limit the spread of the virus. These responses are triggered whether or not the individual has been infected with the pathogen before. Adaptive immune responses are virus-specific, providing the host immune protection from the viral infection in future. These responses provide the individual with ‘memory’ of the previous infection, allowing a rapid and targeted immune response when new exposure to the same or similar viruses occurs. The fundamental objective of vaccination against viral pathogens is to induce adaptive immunity in the naive host, which protects from disease upon any subsequent exposures to the infectious agent. In the absence of vaccine-induced immunity, the initial control of a viral infection depends on mechanisms that constitute the innate immune system, such as production of interferon-α (IFNα) or lysis of virus-infected cells by natural killer cells. Innate immunity limits viral spread but these defenses are often not sufficient to block symptoms of illness during the interval necessary to elicit adaptive immunity against the virus. In extreme circumstances, life-threatening complications may result in the interim. Adaptive antiviral immunity consists of the clonal expansion of T lymphocytes and B lymphocytes that have the functional capacity to recognize specific viral proteins and to interfere with viral replication and transfer of virions from infected to uninfected cells within the host.

In order to induce adaptive immunity, viral proteins must be processed by dendritic cells or macrophages, which are specialized antigen-presenting cells that mediate the cell surface expression of viral peptides in combination with the class I or class II major histocompatibility complex (MHC) proteins. MHC-restricted antigen presentation creates populations of 'memory' T lymphocytes within the CD4 and CD8 subsets that are primed to synthesize cytokines, such as interleukin 2 (IL2) or interferon-γ (IFNγ), when exposed to the same viral peptide–MHC class I or class II protein complex. Cytokines modulate the inflammatory response, expanding and recruiting antigen-specific cytotoxic T lymphocytes (CTL) to the site of viral infection, and inducing B lymphocytes to produce antibodies of the IgM, IgG, and IgA subclasses, which can bind to proteins made by the pathogen or mediate antibody-dependent cellular cytotoxicity.

Adaptive immunity that protects against viral pathogens can be achieved by inoculation of a naive host with an infectious virus that has been attenuated for its capacity to cause disease, or by exposure of the host to viral proteins administered in a noninfectious formulation. Effective priming of adaptive T lymphocyte and B lymphocyte responses by a viral vaccine is expected to block most or all symptoms of infection when the host is exposed to the pathogen. The immunogenicity of a vaccine is defined as its capacity to elicit adaptive immunity, whereas protective efficacy refers to the prevention of disease, which is a consequence of the effective induction of virus-specific immunity. Vaccine-induced immunity may not prevent asymptomatic or abortive infection during these encounters, but memory, or 'recall', responses to the viral proteins should eliminate any serious morbidity or risk of mortality known to be associated with the infection in a susceptible, nonimmunized individual. Antiviral responses elicited by vaccination provide 'active', as distinguished from 'passive' immunity. Passive antiviral immunity is provided by virus-specific IgG antibodies, which may be acquired transplacentally by infants, or by administration of immunoglobulins, such as rabies or varicella zoster immunoglobulin. Active immunity, as elicited by effective vaccines, mimics the memory immunity that follows natural infection and is persistent, whereas passively acquired antibodies are metabolized over a half-life of about 4 weeks and protection is transient.

The challenge of designing viral vaccines that elicit adaptive immunity that is sustained and protective can be addressed using several different strategies, which are often dictated by characteristics of the pathogen and the target population requiring protection. Historically, variolation against smallpox was the first attempt to induce active immunity against a virus by inoculation, as described in early texts from China. Nevertheless, variolation differs from vaccination because unaltered variola virus was given, with disease modification presumed to result from the administration of a low infectious inoculum by a cutaneous route. The first success of viral vaccination is attributed to Benjamin Jesty, an English farmer, who used cowpox to prevent smallpox in 1774, as recounted by Edward Jenner, who published his own experience with vaccination in *Variolae Vaccinae*, 1798. The word 'vaccination' (Latin: vacca for cow) was first used by Jenner because he inoculated material from lesions on the hands of milkmaids that he knew were caused by contact with infected cows, and can be presumed to be cowpox. Successful vaccination was done with viruses from various sources until vaccine preparations began to be standardized in the 1960s. The viruses that are referred to as vaccinia virus strains are orthopoxviruses but are genetically distinct from both cowpox and variola (smallpox) viruses. Their origin remains a mystery. Two hundred years after Jenner's work, the remarkable achievement of the global eradication of smallpox was accomplished using vaccinia virus vaccine. Many viral diseases can now be prevented by immunization and efforts are in progress to make new vaccines that will provide effective prophylaxis against many other human viral pathogens, or in some cases, when given as 'therapeutic' vaccines, which are intended to control the progression of chronic viral infections, such as human immunodeficiency virus (HIV). Vaccination is also used to control viral diseases in nonhuman species.

Live Virus Vaccines

Live attenuated virus vaccines are now licensed in the United States and elsewhere for the prevention of measles, mumps, rubella, varicella, polioviruses 1, 2, and 3, influenza A and B, and rotavirus (**Tables 1** and **2**). When conditions of special risk for exposure exist, live attenuated yellow fever vaccine, live adenovirus, and vaccinia are given as prophylaxis. Live virus vaccines contain an infectious virus as the primary component, which has been attenuated in order to reduce or eliminate its potential to cause disease in the naive host. Vaccine strains are made from RNA viruses, including measles,

Table 1 Live virus vaccines for prevention of human disease

Current	*Under development*
Measles	West Nile virus
Mumps	Respiratory syncytial virus
Rubella	Parainfluenza viruses 1, 2, 3
Varicella	Herpes simplex viruses 1 and 2
Polioviruses 1,2,3	Cytomegalovirus
Yellow fever	Dengue virus
Adenovirus	
Vaccinia	
Rotavirus	
Influenza A and B	

Table 2 Noninfectious vaccines for prevention of human viral diseases

Current	*Under development*
Polioviruses 1,2,3	SARS virus
Influenza A and B	Human immunodeficiency virus
Hepatitis A	Herpes simplex viruses 1 and 2
Hepatitis B	Respiratory syncytial virus
Japanese encephalitis virus	Hepatitis C
Tick-borne encephalitis	West Nile virus
Rabies virus	Ebola virus
Human papillomavirus	
H5N1 avian influenza	

mumps, rubella, poliovirus, rotavirus, influenza, and yellow fever as well as DNA viruses, such as varicella, adenovirus, and vaccinia. Attenuation of virulence is accomplished by laboratory manipulations of the naturally occurring, wild-type virus, which is referred to as the parental strain of the vaccine virus. The parental strains of live attenuated virus vaccines are obtained from an individual experiencing the typical disease caused by the virus. Alternatively, attenuation is achieved by taking advantage of host range differences in virulence between human and closely related animal viruses. Proteins made by the animal virus are similar enough to those encoded by the human pathogen to elicit protective, adaptive immune responses. When a suitable animal model is available, a reduction in the capacity of the vaccine virus is demonstrated and an alteration in the potential to cause disease may be documented. Although strain selection and characterization can be done *in vitro* and in animal models, to predict safety, sequential evaluation of vaccine strains in individuals who have natural immunity, followed by gradual dose escalation studies in susceptible individuals is required to prove safety for human use.

The attenuation of a live vaccine strain is defined clinically by a loss in its potential to cause disease. The attenuated virus should retain infectivity at the site of inoculation, which may be by subcutaneous injection or by oral or intranasal delivery to mucosal cells. In order to be attenuated, the tropisms of the parent virus that would otherwise allow it to produce damage to the host must be incapacitated. For example, the attenuation of polioviruses requires that the vaccine strains be incapable of infecting cells of the central nervous system. Some recipients may have reactions to live attenuated vaccines including fever, but the incidence of reactogenicity such as fever is low and other manifestations such as rash are mild.

The attenuation of RNA and DNA viruses to produce vaccine strains used in most licensed vaccines is accomplished by traditional approaches in which the parent virus undergoes passage *in vitro*, using human or nonhuman cells, or sequentially in both human and nonhuman cells, or by growth in chick or duck embryo cells in eggs. An additional strategy for achieving attenuation is to modify environmental conditions, such as adapting the virus to grow at low temperatures. Cold-adapted viruses are less able to replicate at human body temperature. Measles vaccine is made by passage into chick embryo cells, with further attenuation achieved by cold passage at 32 °C. Rubella vaccine, RA27/3 strain, is derived by passage into human cells only, including growth at 30 °C. Varicella vaccine was attenuated by passage into guinea pig embryo cells and cold passage. These methods create selective pressure for the emergence of mutants that replicate more effectively under the particular tissue culture conditions but are not favored during natural infection in the human host. Because the modern techniques for genetic engineering of viral genomes were not available when these vaccines were developed, their attenuation was determined in clinical trials. These clinical trials established whether the tissue culture passage had yielded viruses that were attenuated in that they did not cause disease but were not so attenuated that immunity was not induced. The molecular basis for the attenuation of most of these vaccines remains undefined because the vaccine preparations typically contain mixtures of viral genomes with varying mutations, and genomes may have multiple changes.

The master donor vaccine virus for the live attenuated influenza vaccine (LAIV) was developed from serial passage into chick kidney cells at sequentially lower temperatures. The end result was acquisition of mutations that conferred the cold-adapted, temperature-sensitive, and attenuated phenotype. In contrast to wild-type influenza virus, the vaccine virus replicates efficiently at 25 °C and does not replicate efficiently at 39 °C (type A strains) or 37 °C (type B strains). The modifications result in a vaccine virus that replicates efficiently in the nasopharynx (colder environment) to initiate immune responses via IgG and mucosal IgA antibodies but replicates poorly in the lower airways and lung (warmer environment), avoiding severe influenza infection.

Attenuation is achieved by using a combination of approaches for the pentavalent rotavirus vaccine. Rotavirus is the most important etiologic agent of severe diarrhea in children less than 5 years of age. Rotavirus causes approximately 440 000 deaths and 2.3 million hospitalizations every year in the world. Taking advantage of host range differences, the vaccine is based on a bovine rotavirus strain, which is naturally attenuated for humans but not broadly cross-protective. Molecular techniques are then used to reassort genes from the bovine rotavirus with genes that encode the major outer capsid proteins from the most common human rotavirus serotypes. As a consequence of these manipulations, the vaccine virus remains infectious but its ability to replicate in the human host is limited and the cycles of viral replication that occur in the vaccine recipient do not result in a reversion to virulence.

Similarly, reversion to wild type for the LAIV is highly improbable as multiple changes (five loci on three gene segments for donor influenza A virus and three loci on two gene segments for donor influenza B virus) of the mutations would have to occur concurrently. In contrast, reversion to wild-type virus with the Sabin vaccine strains in oral polio vaccine is observed. The rate of vaccine-associated poliomyelitis is 1 per 750 000 recipients, and it occurs secondary to reversion to virulence. Though mutations in other regions have an attenuation effect, mutations in the 5′ noncoding region of the genome have been identified to confer attenuation, and a single base change (reversion of the mutation) has been found in virus isolated from vaccine recipients with vaccine-associated poliomyelitis.

By definition, the vaccine strain must retain genetic stability in order to preserve its attenuation. Sequence differences from the parent strain have been implicated in the attenuation of poliovirus strains 1, 2, and 3 that are used to make live poliovirus vaccines. However, the genetic basis for the attenuation of most traditional vaccine strains is not known. The evidence that these strains are genetically stable is inferred from the preservation of the attenuation phenotype when the vaccine is given to susceptible individuals. Even when sequence information is available for vaccine strains, it is difficult to determine which sequence differences from the parent strain are most essential for the biologically observed modification of virulence. The definition of genetic markers of attenuation is complex because the traditional procedures for making live vaccines typically yield many variations in the genome sequence of the vaccine strain and genetic stability can be predicted to be multifactorial. In general, genetic stability is enhanced as the number of mutations in the vaccine strain increases. For example, the vaccine poliovirus type 1 has 56 mutations in 7441 nucleotides compared to only 10 of 7429 differences in the type 3 strain. Live attenuated vaccines may also contain mixed populations of the vaccine virus that have different genetic alterations, as has been described for rubella vaccine. In most cases, biological attenuation means that the vaccine virus also loses its transmissibility to other susceptible individuals who are in close contact with the vaccine recipient. However, when vaccine strains are transmissible, the genetic stability of the vaccine virus must also be preserved after replication in secondary contacts.

The immunogenicity of live attenuated virus vaccines depends upon the selection of an appropriate infectious dose for inoculation, whether the vaccine is given by systemic or mucosal routes. For example, a high-potency varicella zoster virus vaccine is approved for adults greater than 60 years of age to reduce the risk of herpes zoster and postherpetic neuralgia. The infectious virus content in the zoster vaccine is >14-fold higher than the VZV vaccine used for routine childhood immunizations. Herpes zoster is associated with a decline in cell-mediated immunity to VZV. The higher potency vaccine boosts the cell-mediated immunity of adults greater than 55 years of age, and clinical trials demonstrated a reduction in morbidity from herpes zoster and postherpetic neuralgia. The identification of the proper dosage regimen for administration is also important. Many live attenuated virus vaccines must be given as several doses in order to establish persistent adaptive immunity in the majority of naive recipients. Some live virus vaccines consist of mixtures of vaccine strains because protection must be conferred against disease caused by different subgroups of the wild-type virus, as illustrated by the trivalent oral poliovirus vaccine. In other cases, several live attenuated virus strains are combined to facilitate simultaneous immunization of the susceptible host against unrelated viruses, as exemplified by the measles–mumps–rubella (MMR) vaccine. The challenge of designing these multivalent vaccines is to ensure that each attenuated vaccine strain is present in a high enough inoculum to allow it to replicate adequately at the site of inoculation in the presence of the other strains. For example, the combined live attenuated measles, mumps, rubella, and varicella (MMRV) vaccine has a higher titer of Oka/Merck varicella zoster virus as compared to the single-antigen varicella vaccine, whereas the titers for measles, mumps, and rubella in MMRV vaccine are identical to titers in the MMR vaccine. A balance of the components must be achieved to prevent interference by more potent vaccine viruses that might impair the immunogenicity of the other vaccine strains. The establishment of adaptive immunity to all components may depend upon a multiple-dose regimen, as is recommended for live attenuated polio vaccine. The timing between doses of live attenuated virus vaccines is also important because interference can occur when one live virus vaccine is given too soon after another. The required interval is usually at least 4 weeks, to avoid reduction in the infectivity of the second vaccine strain as a result of the replication of the first vaccine strain or as a result of antiviral immune responses elicited by the first vaccine, such as interferon production.

Healthy young children constitute the primary target population for the live attenuated vaccines to prevent measles, mumps, rubella, varicella, polioviruses 1, 2, and 3, influenza A and B, and rotavirus. In contrast, the live yellow fever and adenovirus vaccines are used in individuals who are considered to be at particular risk. Yellow fever vaccines are used to prevent the disease in local populations and in visitors to endemic areas. These vaccines are made from a strain first developed in the 1930s, which was attenuated by passage into monkeys and then prolonged tissue culture passage. The vaccine strain causes a low level of viremia, which is also characteristic of infection with the wild-type virus, but multiple sequence changes from the parent strain have been demonstrated, and clinical experience demonstrates that its pathogenic potential to cause life-threatening dissemination is eliminated. Adenovirus vaccines against serotypes 4 and 7 have been used to control outbreaks among military recruits. Prevention of respiratory tract infection is achieved by the oral administration of live adenovirus in tablets that are coated to prevent acid inactivation in the upper gastrointestinal tract. In this instance, attenuation results from the route of inoculation without any molecular alteration of the viral genome.

Noninfectious Vaccines

Noninfectious vaccines are licensed for influenza, polio, hepatitis A, hepatitis B, rabies, Japanese encephalitis virus, tick-borne encephalitis, and human papillomavirus (HPV) (**Table 2**). The vaccines are made by inactivating infectious virus after growth in tissue culture or eggs, or by using only the protein components of the virus. These vaccines are referred to as ‘killed’ or ‘inactivated’ vaccines, or as ‘subunit’ vaccines. This approach to vaccine design has the advantage of eliminating concerns about the infectious component of attenuated live viruses. While attenuation of virulence is the major issue in making live virus vaccines, immunogenicity is the primary concern in designing inactivated vaccines. Alum is used as an adjuvant to provide amplification of adaptive immunity, which is achieved by viral replication in the case of live attenuated vaccines. The induction of a balanced host response against viral proteins is of critical importance in the production of inactivated vaccines, as illustrated by the occurrence of atypical measles disease in children who were immunized with a formalin-inactivated, alum-precipitated measles vaccine. Formalin-inactivated respiratory syncytial virus vaccine was associated with severe lower respiratory tract infection in immunized infants who were infected with the wild-type virus. Although formalin inactivation creates safe inactivated vaccines for other viral pathogens, cross-linking by formaldehyde may have changed the conformation of viral proteins, inducing antibodies against amino acid epitopes that were not elicited in the normal host response to viral proteins made during replication in host cells. Immunization with inactivated measles vaccine appears to have resulted in the formation of antigen–antibody immune complexes when viral infection occurred. This misdirection of the adaptive immune response resulted in immune-mediated disease, instead of protective immunity, in some vaccine recipients. Since inactivated vaccines are not as immunogenic for inducing memory host responses as natural infection or live virus vaccines, most dose regimens for inactivated or subunit vaccines incorporate ‘booster’ doses to ensure the long-term persistence of virus-specific immunity.

Inactivated influenza vaccine is used to protect individuals who are at risk for life-threatening disease during the annual epidemics of influenza A and B. The target populations for this vaccine are elderly adults, immunocompromised patients, and those with chronic pulmonary or cardiac diseases. Because influenza viruses undergo rapid antigenic changes, it is necessary to formulate the vaccine annually to contain the hemagglutinin (HA) and neuraminidase (NA) proteins from the two predominant circulating strains of influenza A and the major influenza B strain, which are identified through a global surveillance network. The component viruses are grown in embryonated eggs, inactivated by formalin, and combined in a trivalent vaccine. Subunit preparations of influenza vaccine are made by detergent treatment to increase relative concentrations of HA and NA proteins. In the case of influenza vaccine, the need for repeated immunization is dictated by the genetic capacity of influenza viruses to undergo antigenic drift and shift, requiring administration of the new vaccine to high-risk populations before each winter epidemic begins. Inactivated polio vaccine is also a trivalent vaccine made from formalin-inactivated strains of polioviruses 1, 2, and 3. The manufacture of inactivated polio vaccine is complicated by the need to achieve complete inactivation of these nonattenuated viruses while maintaining immunogenicity that is protective against paralytic disease caused by each of the three polio serotypes. The current enhanced potency vaccine given as five doses, beginning in infancy with later booster doses, is now recommended as an alternative to live attenuated polio vaccine in developed countries. Regimens combining initial immunization with inactivated vaccine followed by doses of live attenuated vaccine are also effective. Inactivated polio vaccine must be given by injection, which is a practical limitation to its use in developing countries.

Whereas most viral vaccines are designed to prevent the disease caused by acute primary infection, the benefit of hepatitis B vaccine results from preventing chronic active infection and the late sequelae of hepatic failure and hepatocellular carcinoma. In contrast to influenza and polio, hepatitis B virus does not replicate in tissue culture. The vaccine for hepatitis B consists of the surface antigen of the virus, which is a glycoprotein that forms the outer envelope of the virion. When expressed by introducing the gene sequence into yeast or mammalian cells, the hepatitis B surface antigen self-assembles into a particle structure, which contributes to its immunogenicity as a single viral protein, and allows its use as an effective single-protein subunit vaccine. Recombinant DNA vaccines have replaced vaccines in which surface antigen particles were extracted from plasma of chronic carriers. The success of hepatitis B vaccine depends in particular upon the timely delivery of the vaccine to infants. Vaccination beginning at birth blocks the transmission of hepatitis B virus from carrier mothers to their infants, who are otherwise at high risk for chronic infection. Although both viruses cause hepatitis, hepatitis B is a DNA virus while hepatitis A is an enterovirus belonging to the same family as polioviruses. New vaccines for hepatitis A that contain formalin-inactivated virus grown in tissue culture have been licensed and are administered with alum or liposomal adjuvants. Hepatitis A vaccine is recommended for universal administration to children at 1 year of age in the United States, where the cost of vaccination is acceptable even though the risk of serious disease in young children is low. Hepatitis A vaccine is useful for susceptible adults who may be exposed due to occupation, travel, and other risk factors, as well as during community outbreaks.

Like the hepatitis B vaccine, the HPV vaccine is designed to prevent chronic infection and subsequent development of cancer, specifically, cervical, vaginal, and anal cancer. Cervical cancer is the second most common cancer in women worldwide and third most fatal, killing 290 000 women per year. Studies have proven that HPV infection precedes development of cervical cancer. Two structural proteins, L1 (major component) and L2, make up the capsid of papilloma virus, a nonenveloped double-stranded DNA virus. The L1 major capsid protein is the antigen used in HPV vaccines. For the quadrivalent HPV vaccine licensed in the United States, L1 protein is produced via recombinant DNA technology. A high-yield *Saccharomyces cerevisiae* (yeast) system produces L1 proteins that self-assemble into conformationally intact noninfectious virus-like particles. Each quadrivalent HPV vaccine includes L1 proteins from HPV type 16 and 18, which are the most common oncogenic types, and from HPV type 6 and 11, which are the most common genital wart types.

Inactivated vaccines are licensed to prevent three viral causes of central nervous system disease, including rabies, Japanese encephalitis virus, and tick-borne encephalitis. In 1885, Louis Pasteur inoculated Joseph Meister with spinal cord material from infected rabbits that was inactivated by drying but contained some infectious virus. This work initiated a vaccine method based upon use of nervous tissue from infected animals that continued to be used during the first half of the twentieth century, with later modifications made to improve viral inactivation. The current rabies vaccine is made from virus grown in human cells in tissue culture and inactivated with β-propiolactone, which eliminates the risks of adverse reactions to myelinated animal tissues. The administration of the vaccine to exposed individuals is simplified to a five- or six-dose regimen instead of the 14–23 doses required for earlier rabies vaccines. Whereas most viral vaccines protect against infections acquired by human to human transmission, immunization of domestic animals with inactivated rabies vaccine is critical for disease prevention. Japanese encephalitis virus is a flavivirus, related to St. Louis encephalitis virus and other members of this family, which is maintained as a mosquito-borne pathogen in Asia. Although most infections are asymptomatic, some individuals develop encephalitis that is fatal or causes severe, permanent neurologic damage. The licensed vaccine for Japanese encephalitis is an inactivated preparation purified from infected mouse brain although inactivated and live attenuated vaccines made in tissue culture are used in China. The need for immunization is restricted to populations in endemic areas and for travelers who are visiting rural areas in these countries during the peak season for transmission in summer and fall. Tick-borne encephalitis virus is also a flavivirus, with subgroups called Far Eastern and Western virus types. The distribution of endemic areas includes parts of Europe and Russia. The vaccine used in Europe is made from formalin-inactivated virus grown in chick embryo cells. Immunization is recommended for populations in endemic areas and for travelers to these areas who may have increased risk of exposure to ticks.

Vaccine Immunology

Because the development of new viral vaccines takes years and is very costly, immunologic criteria are used to judge the probable efficacy of candidate vaccines. Laboratory assays for assessing vaccine immunogenicity measure the production of antibodies directed against viral proteins, including IgG and secretory IgA antibodies, as well as their functional capacity to neutralize the virus *in vitro* or to mediate antibody-dependent cellular cytotoxicity. Because of the importance of cell-mediated immunity for defense against viral infections, assays for cytokine production by T cells stimulated with viral antigens *in vitro* and for T-cell-mediated cytotoxicity are useful measures for the establishment of virus-specific memory immunity. Establishing accurate correlates of protection must be done in field trials during which large cohorts of vaccinees are exposed to wild-type virus. In most instances, only simple laboratory assays can be performed when so many individuals must be tested. Serologic assays are used for this purpose even though protection is likely to require adequate T-cell-mediated immunity. It is necessary to have a reliable expected attack rate for transmission of the viral pathogen whereas, under field conditions, rates of transmission are affected by many variables, such as the proximity and duration of contact with the index case. Therefore, most clinical vaccine trials require large populations of subjects. Whether protective immunity is induced by viral vaccines is often proved conclusively only after widespread implementation of immunization programs, as illustrated by the impact of vaccines against childhood diseases, such as measles, mumps, and rubella.

The specific goals of vaccine immunology are to demonstrate that vaccination induces adaptive immunity against relevant viral antigens in the naive host and, when possible, to identify immunologic responses that are associated particularly with protection of vaccine recipients against the usual consequences of infection with the wild-type virus. In practice, the definition of immunologic correlates of vaccine protection is rarely straightforward. The immunogenicity of vaccines is usually assessed by comparison with immune responses that follow natural infection with the same virus but, in most instances, specific correlates of protection are not known for naturally acquired immunity. The redundancy of the mammalian immune system means that a broad range of adaptive immune responses to the pathogen can be measured in the healthy immune individual. For example, individuals who have antibodies to viral proteins can also be expected to have antigen-specific CD4+ and CD8+ T cells. Vaccine-induced antibodies, especially those with neutralizing activity against the virus, have been considered the first line of defense against infection when the immunized host encounters the wild-type virus. These antibodies may limit initial replication at the site of viral inoculation. Primary vaccine failure is defined as a failure of the initial doses of the vaccine regimen to induce virus-specific antibodies. Effective vaccines are expected to elicit seroconversion in most vaccine recipients, which often requires the administration of several doses. Nevertheless, seroconversion is not invariably a predictable marker of protective immunity. Immunization usually induces a range of antigen-specific antibody responses in different individuals. In some cases, detection of any antibodies to viral proteins correlates with protection whereas in other cases, a 'protective' titer is defined as greater than or equal to a particular concentration of antibodies. The appropriate laboratory marker of protection may also differ depending upon the nature of the vaccine that is being evaluated. For example, inactivated vaccines often elicit high titers of virus-specific antibodies that correlate with protection while live attenuated vaccines are more likely to induce cellular immunity and lower antibody titers. Despite these differences, the inactivated and live attenuated forms of vaccine may be equally effective against the same pathogen. Live attenuated virus vaccines often induce a more persistent cell-mediated immune response, which affords protection even when antibody titers fall below the threshold of detection in standard serologic assays. In the case of live virus vaccines, rates of vaccine virus shedding after the inoculation of naive subjects may be as reliable a marker of protection as immunologic assays. Depending upon the assessment of risk, correlates of vaccine protection may be defined by deliberate direct challenge of immunized volunteers with the wild-type virus.

Whether or not precise correlates of protection can be defined, immunologic assays are useful for demonstrating effects of vaccine composition and host factors on the response to viral vaccines. These analyses provide valuable insights about the effect of age. For example, immunologic studies of live attenuated varicella vaccine revealed that adolescents and young adults require a two-dose regimen to achieve humoral and cell-mediated immune responses that are equivalent to those induced by a single dose in young children. The immunogenicity of vaccines given to young infants may be diminished by transplacentally acquired antibodies, as is observed in measles immunization. Immunologic assays are also useful to determine whether different viral vaccines are compatible when administered concurrently. Since the immunogenicity of viral vaccines in infants is influenced by nutritional status and factors such as the prevalence of intercurrent infections with gastrointestinal pathogens, vaccine formulations that are effective in developed countries may not be appropriate in other circumstances. A need to adjust vaccine dosage or regimen may be evident from comparative immunogenicity studies. Assessing the interval over which adaptive immune responses remain detectable is necessary because waning immunity may indicate the need for booster doses of the vaccine. In addition to primary vaccine failure in which the initial immunogenicity is inadequate to prevent disease caused by wild-type virus, secondary vaccine failures occur when immunity declines over time to nonprotective levels. For example, in the United States, a second booster dose of VZV vaccine is recommended as part of the routine childhood immunization schedule, though it is not known if 'breakthrough' chicken pox cases are predominantly due to primary or secondary vaccine failure. Finally, the control of vaccine-preventable disease often depends not just upon immunogenicity in individual vaccinees but upon achieving adequate levels of herd immunity. Immunologic assays provide information necessary to predict whether the local introduction of the virus is likely to be sustained through secondary transmissions and result in a community outbreak.

Molecular Approaches to Viral Vaccine Design

The advances in molecular biology that have occurred during the past several decades have generated new opportunities for making viral vaccines. Molecular approaches to the designing of human viral vaccines will have a major impact in helping to address deficiencies of existing vaccines and allowing the invention of vaccines against infectious diseases that are not preventable by immunization at this time. Molecular techniques are already being implemented to improve licensed vaccines, as illustrated by the use of cDNA clones to reduce the frequency of poliovirus mutations during vaccine manufacture and the use of reassortment methods to incorporate new influenza viral antigens into available virus strains that replicate to the levels required for production of the inactivated influenza vaccine. Hepatitis B vaccine is now made from recombinant surface antigen protein. Molecular approaches that are being developed to create new or improved vaccines include the genetic engineering of live attenuated virus vaccines, in which targeted mutations or deletions are made in genes that are determinants of virulence or tissue tropism, the synthesis of replication defective viruses as vaccines, the expression of recombinant viral proteins and peptides from plasmids and in constitutively expressing mammalian cells, the synthesis of virus-like particles from viral proteins made in the absence of the viral genome, the administration of 'naked DNA' corresponding to viral genome sequences that are immunogenic, and the use of attenuated human viruses or host range mutants as vectors for expressing genes from unrelated viruses. For example, gene reassortment is used for generating the pentavalent rotavirus vaccine by combining genes encoding human proteins with genes from the donor bovine rotavirus strain and also for generating the LAIV by combining the six internal gene segments from the attenuated donor virus with the two gene segments encoding the relevant wild-type HA and NA proteins. Strategies such as creation of virus-like particles via recombinant DNA technology allow vaccination against viral pathogens such as human papilloma virus, which cannot be grown in tissue culture. In preparation for an influenza vaccine, reverse genetics technology has been optimized to create custom-made influenza strains for use in manufacturing influenza vaccine. The process of reverse genetics is based on inserting the relevant HA and NA genes from wild-type virus and the six internal genes from donor virus into eight plasmids, which are cotransfected in mammalian cells to produce the desired influenza virus strain. The advantage of reverse genetics is its faster more direct creation of the new influenza virus strain compared to the traditional cumbersome approach of sorting through hundreds of influenza strains for the desired HA, NA, and internal gene combinations. Reverse genetics also enables removal of segments of HA and NA genes that encode virulence phenotypes prior to splicing into plasmids (**Table 3**).

Table 3 Molecular approaches for the designing of human viral vaccines

Genetically engineered attenuation
Genome reassortants
Host range variants
Replication-defective viruses
Recombinant viral vectors
Recombinant proteins and peptides
Virus-like particles
DNA vaccines

Molecular methods will also be valuable to redesign current vaccines; for example, the use of DNA vaccines may diminish the interference with measles vaccine immunogenicity associated with transplacentally acquired maternal antibodies. Progress is also being made in the creation of novel adjuvants such as cytokines or immunostimulatory DNA sequences that modulate the host response to enhance antiviral immunity. Newly developed avian (H5N1) influenza vaccines incorporate novel adjuvants that boost the immune response to the vaccine's active constituent thus allowing use of less viral antigen in the vaccine and potentially extending vaccine supplies. The US FDA has approved an inactivated H5N1 influenza virus vaccine, which will only be purchased by the federal government for inclusion in the National Stockpile for future distribution if necessary.

Public Health Impact

The ultimate success of a viral vaccine is realized when the implementation of vaccine delivery programs results in a global reduction of the disease burden caused by the pathogen. The standard set by the smallpox vaccine campaign provides a challenge to eradicate other viral diseases that continue to cause serious disease and death. The worldwide control of measles and polio are the current priorities for eradication initiatives. When the Global Polio Eradication Initiative was launched in 1988, more than 125 countries had endemic wild poliovirus, paralyzing more than 1000 children every day. At the end of 2003, indigenous poliovirus was endemic in only six countries and less than 800 children were paralyzed that year. Even when effective vaccines are available, the need to vaccinate very high percentages of the susceptible population in order to block transmission presents an obstacle to disease control. Viral vaccines are often labile unless frozen, necessitating an intact 'cold chain' during transport to remote areas, and sterile needles and syringes must be available. Mass vaccine campaigns supported by funds from international agencies provide a practical response to these problems through 'National Immunization Days', as was demonstrated by the successful administration of polio vaccine to millions of children in India in a single day.

The opportunity for reducing disease burden by immunization depends on the viral pathogen, how it is transmitted, and the pathogenic mechanisms by which it causes disease. Viral pathogens have evolved concurrently with the human host so that persistence in human populations is assured. Smallpox eradication succeeded by case identification and vaccination of close contacts, but polioviruses circulate by causing asymptomatic infection in most individuals. The cycle of transmission of these viruses may be broken by achieving high levels of vaccine immunity through several summer–fall seasons. Measles is expected to be difficult to eradicate because it is highly contagious, requiring only a few susceptibles in the population to cause an outbreak. Some viruses, most notably the herpesviruses and HIV cause a lifelong persistent infection, associated with intermittent or chronic viral shedding. Control of these viruses differs from those that cause acute infection because reintroduction of the virus into the population can occur readily. HIV presents the exceptionally difficult problem of marked antigenic diversity and rapid emergence of virus subpopulations that can escape adaptive immune responses.

Despite these obstacles, vaccine strategies are essential to reduce the impact of viral diseases because of the limited availability of effective antiviral drugs for most viruses, their short-term efficacy in many circumstances, and the relative cost of antiviral drugs compared with vaccines. The global impact of viral vaccines on public health is recognized as the most important intervention provided by modern medicine.

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Viroids/Virusoids

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Viroid Classification and Structure
Host Range of Viroids
Viroid Infection
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Glossary

agroinoculation Use of engineered *Agrobacterium tumefaciens* that infect certain groups of plants as a vehicle to transfer experimental DNA into plant cell nuclei for transient gene expression.

biolistic bombardment A technique in which gold particles coated with experimental DNA or RNA are delivered under certain amount of pressure into cells for expression.

BY2 cell A cell line derived from *Nicotiana tabacum* cv Bright Yellow 2. It can be propagated easily in an appropriate culture medium. The cells are fast-growing and nearly translucent. They have been extremely useful for studying viral and viroid replication and for studying basic plant cell biology and gene expression.

phloem A type of vascular tissue consisting of several types of living cells that is responsible for the long-distance transport of nutrients and signaling molecules in plants. It is also used by viroids and many viruses to spread infection within a plant.

plasmodesmata Cytoplasmic channels between plant cells that allow cell-to-cell diffusion of small molecules and selective trafficking of RNAs, proteins, viruses, and viroids.

protoplast A plant cell with its cell wall removed by treatment with cellulase and pectinase, enzymes that remove the major cell wall components cellulose and pectin. Protoplasts are prepared fresh for each experiment from plant materials or cultured cells and have been extremely useful for studying viral and viroid replication and for studying basic plant cell biology and gene expression.

RNA silencing A recently discovered mechanism of gene regulation in many organisms. It is mediated by 20–26 nt small RNAs produced from various RNA and DNA sources and functions in regulating RNA stability and translation as well as chromatin modification underlying numerous developmental processes. It also plays a significant role in microbe–host interactions.

viroid A noncoding and nonencapsidated circular RNA that replicates autonomously without helper viruses in a plant.

virusoid A specific group of satellite RNAs, associated with sobemoviruses, that are circular and assuming similar secondary structures with viroids. They have no protein-coding capacity. They replicate by utilizing helper viral-encoded factors and are encapsidated by helper viral coat proteins.

Abbreviations

ASBVd	*Avocado sunblotch viroid*
CCR	central conserved region
CChMVd	*Chrysanthemum chlorotic mottle viroid*
CEVd	*Citrus exocortis viroid*
CsPP2	PP2 from cucumber
HPII	hairpin II
HSVd	*Hop stunt viroid*
LTSV	*Lucerne transient streak virus*
PLMVd	*Peach latent mosaic viroid*
pol II	polymerase II
PSTVd	*Potato spindle tuber viroid*
RYMV	*Rice yellow mottle virus*

Defining Statement

Viroids and virusoids are the smallest pathogens that infect plants. They are single-stranded, circular RNAs and do not encode any proteins. They present simple models to study how an infectious RNA replicates in a host cell and spreads systemically to cause diseases. They are also excellent models to investigate the basic structure–function relationships of RNAs.

Introduction

Theodor O. Diener was credited with the discovery of the first viroid, *Potato spindle tuber viroid* (PSTVd), in 1971. This viroid is the causal agent of potato spindle tuber disease first described in the 1920s. Extensive research over the past three decades has established viroids as the simplest form of RNA-based infectious agents. All viroids are single-stranded, circular RNAs with sizes ranging from 250 to 400 nucleotides (nt). Differing from viruses, these RNAs do not have protein-coding capacity and are not encapsidated in a protein or membrane shell. They do not require the presence of a helper virus to establish infection. Thus, the viroid genomes and/or their derivatives contain all of the genetic information for direct replication in single cells and systemic trafficking throughout a plant to establish infection.

Depending on viroid–host combinations, an infected plant may or may not develop disease symptoms. Common viroid disease symptoms include growth stunting, leaf epinasty and deformation, fruit distortion, stem and leaf necrosis, and plant death. Since viroids do not encode proteins, viroid diseases must result from direct interactions between viroid genomic RNAs or their derivatives and specific cellular components.

Virusoids are also circular RNAs that are similar to viroids in size and secondary structure. They do not encode any proteins. However, they rely on protein factors encoded by their helper viruses for replication and encapsidation. Virusoids are a special group of satellite RNAs associated with plant viruses.

Recent research has contributed significant insights into the sequence/structural elements in viroids that are critical for various aspects of replication, systemic spread, and disease formation in an infected plant. Knowledge of potential host proteins that assist various stages of viroid infection is also emerging. Much less is known about the biological functions of virusoids. It is proposed that continuing studies on these subviral pathogens should yield valuable insights into the simplest mechanisms of infection in eukaryotic cells and help uncover the basic principles of RNA structure–function relationships.

Viroid Classification and Structure

There are over 30 species of viroids in the current database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi). They belong to two families, Pospiviroidae and Avsunviroidae, with their type members being PSTVd and *Avocado sunblotch viroid* (ASBVd), respectively. Many species have sequence variants. All viroids are listed in **Table 1**, under their respective families and genera. Their sizes and number of sequence variants are also listed.

The distinguishing features of the two families of viroids are summarized in **Table 2**. The members of Avsunviroidae have a highly branched secondary structure (**Figure 1**(**a**)). There is limited sequence or secondary structural conservation among the different species. They all replicate in the chloroplast and have ribozyme activities. The members of Pospiviroidae generally have a rod-shaped secondary structure (**Figure 1**(**b**)) and conserved sequences among some species, replicate in the nucleus, and are generally considered to lack ribozyme activities. Five broad structural domains are defined in the secondary structures of some viroids in Pospiviroidae. These include the left-terminal domain, pathogenicity domain, central domain that contains a central conserved region (CCR), variable domain, and right-terminal domain (**Figure 1**(**b**)).

Host Range of Viroids

Unlike many viruses, viroids have relatively narrow host ranges, each infecting one or a few plant species in the field. Avsunviroidae mostly infect woody species whereas Pospiviroidae mostly infect herbaceous species. There is evidence for the recent expansion of host ranges for many viroids. For instance, PSTVd was long known to infect potato only in the field. It has recently been reported to infect avocado and tomato in the field. Under experimental conditions, some viroids can infect more species. For example, PSTVd can infect *Nicotiana benthamiana* and some of its variants also infect *N. tabacum.* The infected plants usually do not exhibit noticeable symptoms.

Recent studies tested whether weedy plant species characteristic for potato and hop fields, which are not natural hosts for PSTVd and *Hop stunt viroid* (HSVd), can be potential hosts for transmitting and spreading infection of these viroids, respectively. Indeed, when the leaves of 12 weedy species from the potato field and 14 from the hop field were inoculated with viroid RNAs or cDNAs through biolistic bombardment, many species supported replication of these two viroids. Sequencing revealed the presence of many variants of these viroids in different infected species. Therefore, there is always the potential for viroids to invade new species if conditions permit.

Table 1 Current viroid species

Family Genus	*Species name*	*Size (nt)*	*Number of variants*
Pospiviroidae			
Apscaviroid	*Pear blister canker viroid* (PBCVd)	314–316	22
	Grapevine yellow speckle viroid-2 (GYSVd-2)	361–363	6
	Grapevine yellow speckle viroid-1 (GYSVd-1)	187–368	65
	Citrus viroid-III (CVd-III)	291–297	53
	Citrus bent leaf viroid (CBLVd)	315–329	21
	Australian grapevine viroid (AGVd)	369	9
	Apple dimple fruit viroid (ADFVd)	306–307	10
	Apple scar skin viroid (ASSVd)	329–333	8
	Apple fruit crinkle viroid (AFCVd)	368–372	29
	Citrus viroid-I-LSS (CVd-LSS)	325–330	5
	Citrus viroid-OS (CVd-OS)	329–331	4
	Japanese citrus viroid 1 (JCVd)	331	1
Pospiviroid	*Tomato planta macho viroid* (TPMVd)	360	1
	Tomato apical stunt viroid (TASVd)	360–363	8
	Potato spindle tuber viroid (PSTVd)	341–364	133
	Mexican papita viroid (MPVd)	359–360	7
	Iresine viroid 1 (IrVd)	370	3
	Columnea latent viroid (CLVd)	359–456	25
	Citrus exocortis viroid (CEVd)	197–475	151
	Chrysanthemum stunt viroid (CSVd)	348–356	26
	Tomato chlorotic dwarf viroid (TCDVd)	359–360	3
Cocadviroid	*Hop latent viroid* (HLVd)	255–256	10
	Coconut tinangaja viroid (CtiVd)	254	2
	Coconut cadang-cadang viroid (CCCVd)	246–301	11
	Citrus viroid IV (CVd-IV)	284–286	6
Hostuviroid	*Hop stunt viroid* (HSVd)	267–368	206
Coleviroid	*Coleus blumei viroid 3* (CbVd-3)	361–364	3
	Coleus blumei viroid 2 (CbVd-2)	295–301	2
	Coleus blumei viroid 1 (CbVd-1)	248–251	9
	Coleus blumei viroid (CbVd)	295	1
Avsunviroidae			
Pelamoviroid	*Chrysanthemum chlorotic mottle viroid* (CChMVd)	397–401	23
	Peach latent mosaic viroid (PLMVd)	335–351	297
Avsunviroid	*Avocado sunblotch viroid* (ASBVd)	120–251	83
Elaviroid	*Eggplant latent viroid* (ELVd)	332–335	9

The number of sequence variants are obtained by removing duplicate sequences in the database.
Data are obtained from Subviral Database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi).

Table 2 Distinct features of Pospiviroidae and Avsunviroidae

	Family	
Features	*Pospiviroidae*	*Avsunviroidae*
Secondary structure	Rod-shaped	Branched for most members
Replicate site	Nucleus	Chloroplast
Rolling circle	Asymmetric	Symmetric
Ribozyme activity	Uncertain	Yes for all current members
Hosts	Mostly herbaceous species	Mostly woody species

Reproduced from Ding B and Itaya A (2007) Viroid: A useful model for studying the basic principles of infection and RNA biology. *Molecular Plant-Microbe Interactions* 20: 7–20.

The reasons for the narrow host range of a viroid are not clear. Recent studies suggest a possibility that low level of replication and/or inability to traffic between cells contributes to the limited host range for some, if not all, viroids. For instance, none of the known viroids infect *Arabidopsis thaliana* when inoculated onto this plant. In transgenic *A. thaliana* plants expressing the dimeric (+)-RNAs of *Citrus exocortis viroid* (CEVd) and HSVd, species of Pospiviroidae, replication took place. Agroinoculation of *A. thaliana* with CEVd, HSVd, and

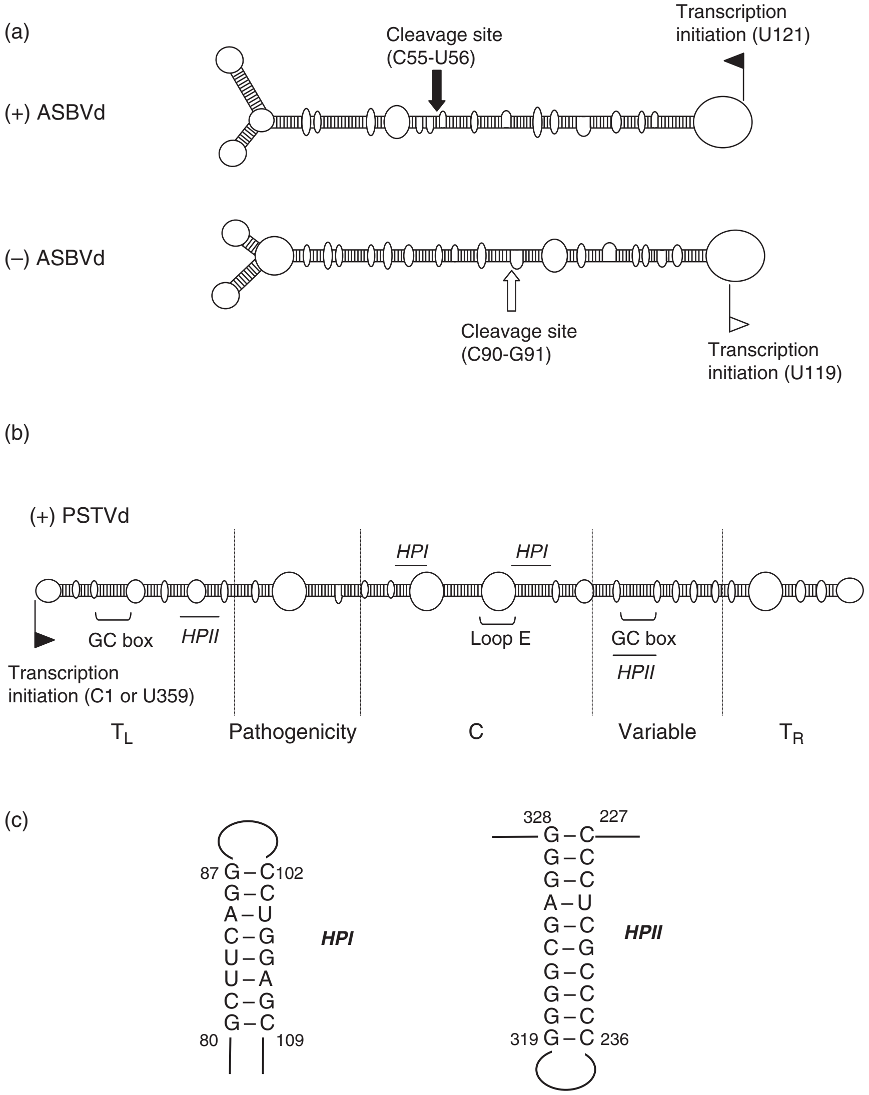
(a)
Transcription
initiation (U121)
Cleavage site
(C55-U56)
(+) ASBVd
(–) ASBVd
Cleavage site
(C90-G91)
Transcription
initiation (U119)
(b)
(+) PSTVd
HPI
HPI
GC box
HPII
Loop E
GC box
HPII
Transcription
initiation (C1 or U359)
T_L
Pathogenicity
C
Variable
T_R
(c)
87 G–C 102
G–C
A–U
C–G
U–G
U–A
C–G
G–C
80 109
HPI
328 227
G–C
G–C
G–C
A–U
G–C
C–G
G–C
G–C
G–C
319 G–C 236
HPII

Figure 1 Secondary structures of ASBVd (a) and PSTVd (b), type members of avsunviroidae and pospiviroidae, respectively. Reproduced, with modifications, from Ding B and Itaya A (2007) Viroid: A useful model for studying the basic principles of infection and RNA biology. *Molecular Plant-Microbe Interactions* 20: 7–20. For PSTVd, the five structural domains are indicated. T_L, left-terminal domain; c, central domain; T_R, right-terminal domain. Arrows indicate the transcription initiation sites and cleavage sites on the viroid genomic RNAs. *HPI* and *HPII* in (b) indicate the positions of nucleotide sequences in PSTVd for the formation of metastable structures HPI and HPII, which are shown in (c). Reproduced, with modifications, from Zhong X, Archual AJ, Amin AA, and Ding B (2008) A genomic map of viroid RNA motifs critical for replication and systemic trafficking. *Plant Cell* 20: 35–47; www.plantcell.org. Copyright American Society of Plant Biologists.

Coleus blumei viroid 1 dimeric cDNAs showed that these viroids did not traffic from the inoculated leaves to distal parts of a plant. For some viroids, their host range may be underestimated if disease symptom is used as the main detection method, because their infection of certain plants will not necessarily produce visible symptoms.

Viroid Infection

Viroid Transmission

Like viral infection in many cases, viroid infection easily spreads between individual plants through wounding of plants caused by farming tools, human contact, or plant–plant contact. Some viroids can also be transmitted via infected seeds or pollens. Vegetative propagation by grafts or tubers also readily transmits viroids. For instance, PSTVd can be transmitted by infected potato tubers and infected tomato seeds. ASBVd can be seed-transmitted in avocado. In contrast to the common transmission of viruses, insect transmission of viroids is not common in the field. There is a report of infrequent transmission of PSTVd by the potato aphid *Macrosiphum euphorbiae*. Quarantine and elimination of infected plant/seed stocks are the most effective means currently available to control the spread of viroid infection.

General Scheme of Systemic Infection in a Plant

The establishment of systemic infection by both families of viroids involves the following mechanistic steps (**Figure 2**): (1) import into specific subcellular organelles (the nucleus for Pospiviroidae and the chloroplast for Avsunviroidae), (2) replication, (3) export out of the organelles, (4) cell-to-cell trafficking, (5) entry into the vascular tissue, (6) long-distance trafficking within the vascular tissue, (7) exit from the vascular tissue and subsequent invasion of nonvascular cells to repeat the cycle. As discussed further below, some steps have been well studied whereas others remain completely unknown.

Intracellular Localization and Replication

Pospiviroidae

In order for replication to take place, members of the family Pospiviroidae must first enter the nucleus. How this is achieved is still poorly understood. The first study to address this question examined nuclear import of fluorescent-labeled *in vitro* transcripts of PSTVd in protoplasts of tobacco BY2 cells. The protoplasts were prepared by the removal of cell walls via digestion with enzymes such as cellulase and pectinase. The protoplasts were further treated with a detergent such as Triton-X 100 to permeabilize the plasma membrane. When the fluorescent-labeled transcripts were incubated with such protoplasts, they entered the cells through the permeabilized plasma membrane and then accumulated in the nucleus within 15–20 min, which was visualized under a fluorescence microscope. When fluorescent transcripts were mixed with a 10 molar excess of nonlabeled transcripts, nuclear import of the former was inhibited. This suggests that PSTVd import is a specific and regulated process, presumably mediated by a protein carrier that remains to be identified. These findings were confirmed with an independent approach, in which PSTVd could function in cis to mediate nuclear import of a large fusion RNA in *N. benthamiana* leaves. Using the latter approach, a recent study showed that the conserved sequence in the upper strand of the PSTVd secondary structure was able to mediate nuclear import of a fusion RNA. The biological significance of this for viroid infection can now be tested. The cellular factor(s) that recognizes and imports the viroid RNA is not known. How the viroid RNAs exit the nucleus remains to be investigated.

Within the nucleus, the viroids replicate via an asymmetric rolling circle mechanism (**Figure 3**(**a**)). Briefly, the circular (+)-RNA is first transcribed into concatemeric linear (−)-strand RNA in the nucleoplasm. This long RNA then acts as the replication intermediate for the synthesis of concatemeric, linear (+)-strand RNA. In one possible mechanism, the latter is transported into the nucleolus, where it is cleaved into unit-length monomers. Subsequent intramolecular end-to-end ligation of each monomer yields the mature, circular progeny viroid RNA. Alternatively, the cleavage and ligation occur in the nucleoplasm, and the mature viroid RNA is transported into the nucleolus for storage.

Several lines of data suggest that the DNA-dependent RNA polymerase II (pol II) is involved in transcription. The purified tomato pol II can transcribe a PSTVd template *in vitro*. The CEVd RNA is associated with the largest subunit of pol II *in vivo*. Treatment of cells or nuclear extracts with pol II inhibitor α-amanitin inhibits replication of CEVd and PSTVd *in vivo* or transcription *in vitro*. In the past few years, a new DNA-dependent RNA polymerase IV and several RNA-dependent RNA polymerases have been discovered in plants. It remains to be tested whether any of these enzymes are involved in viroid transcription.

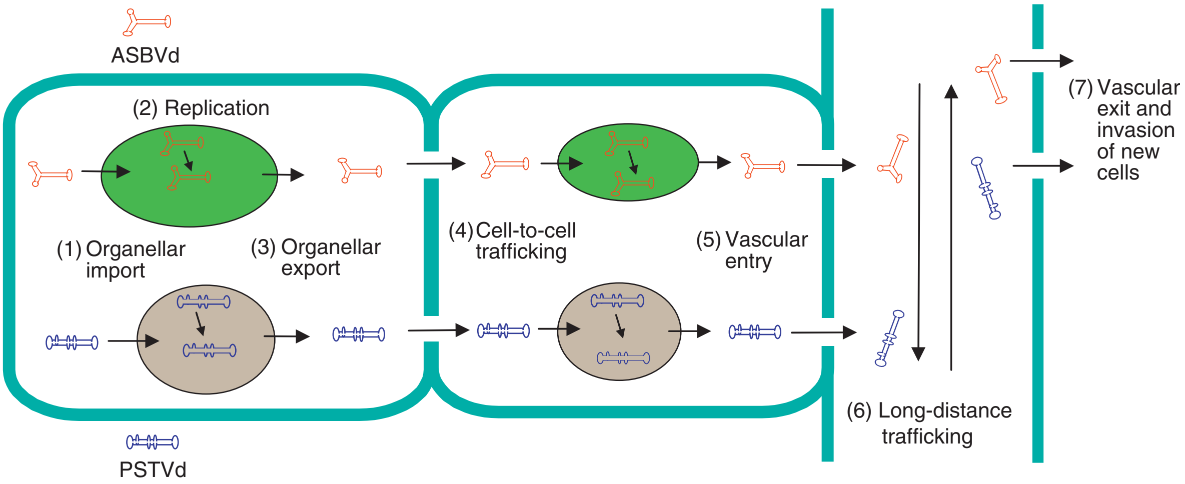
ASBVd
(2) Replication
(1) Organellar import
(3) Organellar export
(4) Cell-to-cell trafficking
(5) Vascular entry
(6) Long-distance trafficking
(7) Vascular exit and invasion of new cells
PSTVd

Figure 2 Distinct steps of systemic infection of ASBVd and PSTVd. Reproduced from Ding B and Itaya A (2007) Viroid: A useful model for studying the basic principles of infection and RNA biology. *Molecular Plant–Microbe Interactions* 20: 7–20.

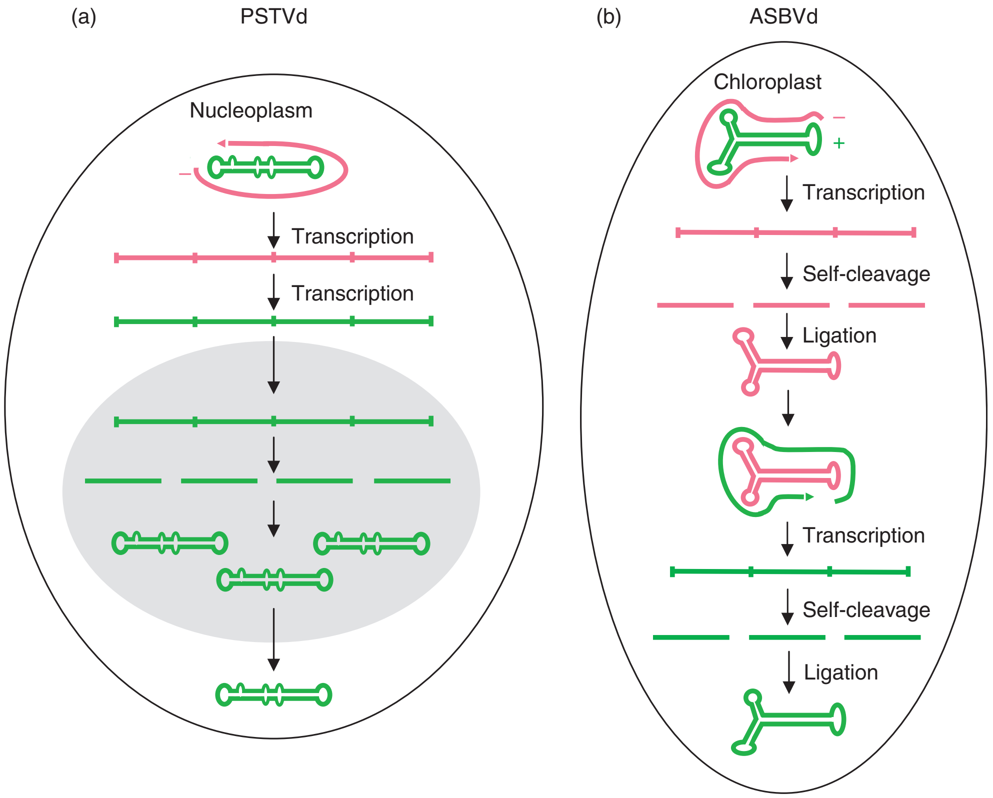
(a)
PSTVd
Nucleoplasm
–
Transcription
Transcription
(b)
ASBVd
Chloroplast
–
+
Transcription
Self-cleavage
Ligation
Transcription
Self-cleavage
Ligation

Figure 3 Rolling circle replication mechanisms of PSTVd (a) and ASBVd (b). The secondary structure sketches of the genomic RNAs illustrate the approximate transcription initiation sites. Reproduced, with modifications, from Ding B and Itaya A (2007) Viroid: A useful model for studying the basic principles of infection and RNA biology. *Molecular Plant–Microbe Interactions* 20: 7–20.

Where transcription initiates in a viroid RNA of the family Pospiviroidae remains to be understood. Recent studies examining the *de novo* synthesis of the (–)-strand PSTVd RNAs in potato nuclear extracts mapped the transcription initiation site on the circular (+)-RNA to U359/C1 of the left-terminal loop (**Figure 1**(**b**)). This can be further tested by loss-of-function genetic experiments in combination with biochemical analysis of where on the viroid RNA the transcription complex binds. The importance of the left-terminal loop for replication was supported by the observation that enlargement of this loop by mutagenesis inhibited replication in protoplasts or infection in a plant in *N. benthamiana.* Furthermore, disruption of each of three consecutive loops from the left-terminal end (loops 2, 3, and 4 in **Figure 4**), respectively, also inhibited replication in protoplasts. The transcription initiation site on the (–)-strand PSTVd template is yet to be identified.

There are two GC boxes in the PSTVd secondary structure (**Figure 1**(**b**)). Mutational studies suggest that they may play a role in transcription. Further studies are

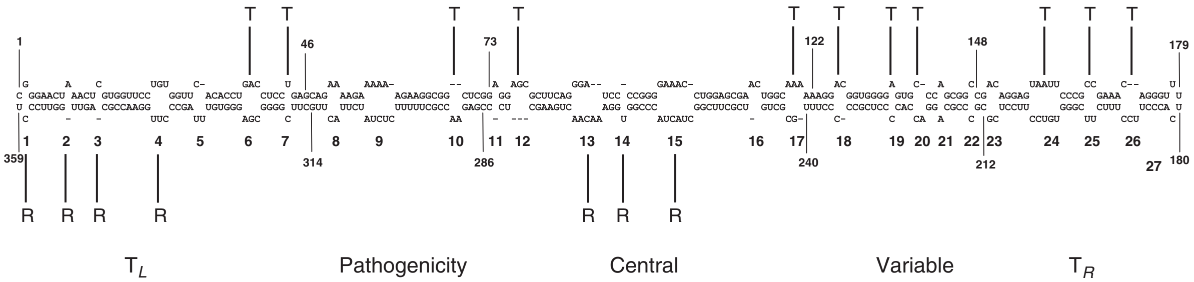
T T T T T T T T T T T
1 46 73 122 148 179
G A C UGU C- GAC U AA AAAA- -- A AGC GGA-- - GAAAC- AC AAA AC A C- A C AC UAAUU CC C-- U
C GGAACU AACU GUGGUUCC GGUU ACACCU CUCC GAGCAG AAGA AGAAGGCGG CUCGG GG GCUUCAG UCC CCGGG CUGGAGCGA UGGC AAAGG GGUGGGG GUG CC GCGG CG AGGAG CCCG GAAA AGGGU U
U CCUUGG UUGA CGCCAAGG CCGA UGUGGG GGGG UUCGUU UUCU UUUUUCGCC GAGCC CU CGAAGUC AGG GGCCC GGCUUCGCU GUCG UUUCC CCGCUCC CAC GG CGCC GC UCCUU GGGC CUUU UCCCA U
C - - UUC UU AGC C CA AUCUC AA - --- AACAA U AUCAUC - CG- C- C CA A C GC CCUGU UU CCU C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
359 314 286 240 212 180
R R R R R R R
T_L
Pathogenicity
Central
Variable
T_R

Figure 4 A genomic map of PSTVd loop motifs critical for replication (R) in single cells or for systemic trafficking (T) in a whole plant. Reproduced, with modifications, from Zhong X, Archual AJ, Amin AA, and Ding B (2008) A genomic map of viroid RNA motifs critical for replication and systemic trafficking. *Plant Cell* 20: 35–47; www.plantcell.org. Copyright American Society of Plant Biologists.

needed to determine their functions. There is evidence that loop E located in the CCR of PSTVd (see **Figure 1**(**b**)) is critical for replication. Recent studies provided evidence that the PSTVd loop E motif exists *in vivo*, has a defined tertiary structure, and disruption of this structure leads to loss of function in replication. A more recent study using whole genome mutational analysis has identified several additional PSTVd loops as motifs critical for replication in single cells. Some loops are located within the central region (loops 13 and 14 in **Figure 4**) and others are located in the left-terminal domain (loops 2, 3, and 4 in **Figure 4**). The specific roles of these loops in RNA stability, nuclear transport, transcription, cleavage, and ligation need to be determined.

A thermodynamically metastable hairpin II (HPII) structure is predicted to form through base interactions involving nucleotide sequences 227–236 and 319–328 (**Figures 1**(**b**) and **1**(**c**)) during thermal denaturation of the PSTVd secondary structure. The HPII structure has been detected *in vitro* and *in vivo*, suggesting its importance in viroid infection. It is also suggested that another metastable structure, HPI (**Figures 1**(**b**) and **1**(**c**)), is important for infection of tomato. However, recent mutational analyses showed that neither HPII nor HPI is critical for PSTVd replication in *N. benthamiana*. Whether these metastable structures function in PSTVd infection in some plant species but not in others is an important question in further studies.

The sequence and structural conservation of the CCR of several members of Pospiviroidae suggests its potential importance in viroid processing. *In vitro* studies mapped the cleavage and ligation site to between G95 and G96 of CCR. In recent work with transgenic *A. thaliana* plants that express dimeric (+)-RNAs of CEVd, HSVd, and *Apple scar skin viroid*, the *in vivo* processing site for these viroids was mapped at equivalent positions of a putative HPI/double-stranded structure formed by the upper strand and flanking nucleotides of the CCR. More specifically, the substrate for *in vivo* cleavage is the proposed conserved double-stranded structure, with HPI potentially facilitating the adoption of this structure, whereas ligation is determined by loop E and flanking nucleotides of the two CCR strands.

It is generally thought that a cellular RNase catalyzes the cleavage of concatemeric RNAs. It has long been known that wheat germ extract and *Chlamydomonas reinhardtii* contain ligase activities that circularize PSTVd linear RNAs. The biochemical identities of any enzymes and associated factors that are responsible for cleavage and ligation of viroids in the family Pospiviroidae remain unknown.

Avsunviroidae

Viroids of the family Avsunviroidae replicate in the chloroplast. How they enter and exit the chloroplast is not known. Within the chloroplast, these viroids replicate via a symmetric rolling circle mechanism (**Figure 3**(**b**)). The circular genomic (+)-RNA is first transcribed into a linear, concatemeric (−)-strand RNA. This RNA is cleaved into unit-length molecules and circularized to serve as the template to generate linear, concatemeric (+)-strand RNA. This RNA is subsequently cleaved into unit-length monomers and circularized. *In vitro* studies showed that the *Escherichia coli* RNA polymerase can transcribe *Peach latent mosaic viroid* (PLMVd) RNA templates *in vitro*, suggesting that the plastid-encoded bacterial-like multiunit RNA polymerase may be involved in transcription *in vivo*. However, sensitivity of ASBVd replication to treatment with targetoxin suggests that the nuclear-encoded and phage-like single-unit polymerase is involved in replication *in vivo*. Further studies are necessary to determine which polymerase is responsible for replication during infection.

The transcription initiation sites have been determined for ASBVd and PLMVd. For ASBVd, *in vitro* capping and RNase protection assays mapped U121 as the initiation site on the (+)-RNA and U119 as the site on the (−)-RNA. Both sites are located in the AU-rich terminal loops of the RNA secondary structures (**Figure 1**). For PLMVd, studies on a wide repertoire of PLMVd variants revealed A50/C51 and A284/A286 as the universal transcription initiation sites for the (+)- and (−)-strand RNAs, respectively. Furthermore, a highly conserved CAGACG sequence appears to be important for defining these sites. The possibility that some variants can start transcription in other sites cannot be formally ruled out.

The viroids in Avsunviroidae form hammerhead ribozymes in both the (+)- and (−)-strands of RNAs to catalyze self-cleavage *in vitro*. The general thought is that these viroids self-cleave during infection *in vivo*. However, there is evidence that cellular factors may enhance this cleavage. UV-crosslinking of viroid RNA–protein complex in infected tissues in conjunction with biochemical analyses identified a chloroplast protein, PARBP33, that interacts with ASBVd *in vivo*. This protein has an RNA-binding motif and accelerates self-cleavage of concatemeric ASBVd RNAs *in vitro*. The *in vivo* role of this factor for viroid replication and general RNA processing remains to be further studied.

Although self-cleavage of both the linear concatemeric (+)- and (−)-RNAs is well demonstrated for members of the family Avsunviroidae, little is known about how monomeric molecules are ligated into circles. Nonenzymatic intra- and intermolecular ligation has been demonstrated for PLMVd *in vitro*. The self-ligation produces a 2′,5′-phosphodiester bond *in vitro*. Recent studies demonstrated the presence of the 2′,5′-phosphodiester bond at the ligation site of the circular PLMVd RNAs isolated from infected peach plants. It will be important to determine whether this mode of ligation functions during PLMVd replication.

A recent technical advance may enable genetic investigation of the cellular factors involved in cleavage and ligation. When the dimeric cDNAs of three species of Avsunviroidae, including ASBVd, *Chrysanthemum chlorotic mottle viroid* (CChMVd), and *Eggplant latent viroid*, are expressed in the transformed chloroplasts of *C. reinhardtii*, the dimeric (+)- or (–)-RNA transcripts are correctly cleaved into unit length molecules and circularized. There is no evidence for replication to have taken place. Given the complete genome sequence information and well-established genetic and molecular approaches in *C. reinhardtii*, this model system may prove to be of great utility to identify the protein factors important for viroid RNA processing.

Viroid Cell-to-Cell and Long-Distance Trafficking

To establish a systemic infection, viroid RNAs must traffic from initially infected cells into neighboring cells and distant organs. Studies on PSTVd indicate that cell-to-cell trafficking occurs through cytoplasmic channels plasmodesmata and long-distance trafficking occurs through the vascular tissue phloem. Without encoding proteins, one can assume that viroids either diffuse between cells and through the phloem or they have sequence/structural motifs to mediate the trafficking process. The first clue for motif-mediated trafficking came from studies showing that two mutations in the right-terminal domain of PSTVd do not appear to affect replication in tomato roots, but affect systemic infection. Further studies with microinjection showed that PSTVd could function in cis to potentiate cell-to-cell trafficking of a heterologous RNA, suggesting that the viroid RNA has a motif that mediates trafficking. Furthermore, in an infected flower PSTVd traffics into sepals but not the other floral organs, suggesting that the phloem has a mechanism to recognize and traffic PSTVd RNAs into selective sink organs.

Mutational studies on two PSTVd strains, PSTVd^{NT} and PSTVd^{NB}, which differ by 5 nt, identified a bipartite motif that is required for trafficking from bundle sheath to mesophyll, but not required for trafficking in the reverse direction. Importantly, this motif is required for trafficking in young leaves, but not in mature leaves. Thus, plant development is also a major factor for the fine-tuning of trafficking controls. Whether the bipartite motif interacts with separate cellular factors or they form a particular tertiary structural motif via conformational changes to interact with a cellular factor for trafficking remains an outstanding question.

A tertiary structural motif, which consists of at least U43/C318 (loop 7 in **Figure 4**) that interacts with *cis*-Watson–Crick base pairing with water insertion, has been shown to be required for PSTVd to traffic from the bundle sheath into the phloem to initiate long-distance transport in *N. benthamiana.* Mechanistically, water insertion distorts the structure of the local helix. This distortion is necessary for trafficking. This motif recurs in many other RNAs. In rRNAs, this motif is a binding site for a ribosomal protein.

These studies imply the existence of multiple PSTVd structural motifs to mediate trafficking across various cellular boundaries in an infected plant. Indeed, a recent study has identified many additional loops in the PSTVd secondary structure that are critical for systemic trafficking in *N. benthamiana* (**Figure 4**). Closing of these loops by nucleotide substitutions/deletions to create Watson–Crick base pairing abolishes systemic infection while allowing replication. The tertiary structure of each loop, whether each of these loops function individually or in some combinations to mediate trafficking across specific cellular boundaries, and what host proteins interact with each of these loops for function are outstanding issues for future investigations.

A current hypothesis is that certain cellular proteins recognize specific viroid RNA motifs to potentiate trafficking between cells and among organs. These proteins have not yet been identified. Several promising candidates have been reported but their functions have to be conclusively tested. These include the mobile phloem lectin PP2 from cucumber (CsPP2) that binds HSVd *in vitro* and *in vivo* as well as two phloem proteins that bind ASBVd. A tomato protein, VIRP1, interacts *in vitro* with the right-terminal region of PSTVd and HSVd. Recent work showed that VIRP1 appears to be important for infection. When its expression is repressed by antisense method in transgenic *N. benthamiana*, the protoplasts prepared from this transgenic plant fail to support PSTVd replication.

Viroid Pathogenecity

Without encoding proteins, viroid diseases must result from interactions between the viroid genome, or genome-derived RNAs, and cellular factors. Such interactions disturb the normal course of plant development leading to disease formation. Viroid diseases show great variations, depending on viroid–host combinations. They range from nearly symptomless to host lethality. One of the most devastating diseases is the cadang-cadang disease that killed over 30 million coconut palms, caused by infection of *Coconut cadang-cadang viroid.* Environmental conditions affect symptom expression. In particular, high temperatures enhance disease symptoms. No natural resistance to viroid infection has been reported.

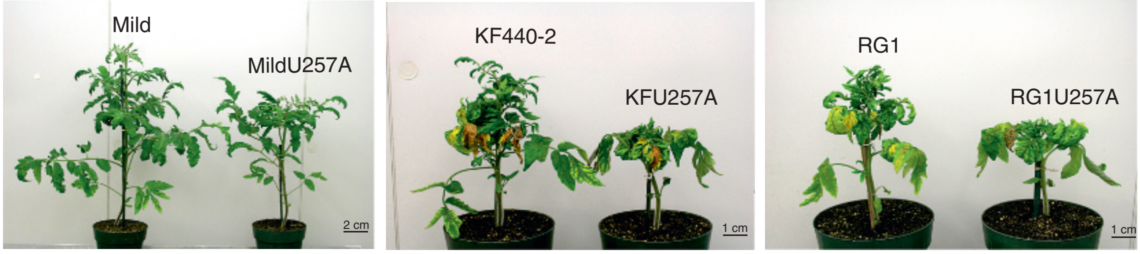

Figure 5 Mild to lethal disease symptoms caused by infection of several PSTVd variants in Rutgers tomato plants. Reproduced from Qi Y and Ding B (2003) Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. *Plant Cell* 15: 1360–1374. www.plantcell.org. Copyright American Society of Plant Biologists.

In many cases, small sequence or structural variations in a viroid genome can cause symptoms of different degrees of severity (**Figure 5**). The viroid RNA structure and disease relationships have been studied most extensively for members of the family Pospiviroidae. Early studies with PSTVd and CEVd showed that many nucleotide changes in association with different degrees of symptom severity occur in the so-called pathogenicity domain (**Figure 1**(**b**) for PSTVd). More recent studies indicate that all five structural domains play a role in pathogenicity.

Sequence comparisons among ASBVd clones isolated from diseased and healthy tissues of infected avocado suggest that a 'U' insertion between nt 115 and 118 in different variants is responsible for the symptoms. Studies on symptomatic and nonsymptomatic variants of CChMVd identified tetraloop UUUC (nt 82–85) as a major pathogenicity determinant. Conversion of this tetraloop to GAAA in natural variants or by mutagenesis renders the viroid nonsymptomatic.

Other than correlations between viroid sequences and symptom severity, little is known about the mechanisms of pathogenicity. In general, viroid replication levels and tissue localizations are not major factors for the varying degrees of symptoms. This suggests that specific molecular interactions between viroid sequences/structures with host factors are prevailing disease mechanisms. The cellular factors that interact with specific viroid sequences/structures for disease development are not known. Infection of tomato by mild and severe PSTVd strains induced or suppressed expression of common and unique sets of host genes. These include genes involved in general defense/stress responses, cell wall structure and metabolism, chloroplast functions, and so on. Similar alteration of host gene expression has also been reported in Etrog citron leaves infected by *Citrus viroid III*. How the altered expression of any host genes contributes to disease formation is not known. PSTVd infection also causes phosphorylation of a protein kinase that is immunologically related to the mammalian interferon-induced, double-stranded RNA-activated protein kinase. Further studies showed differential *in vitro* activation of the mammalian protein kinase P68 by PSTVd strains of different pathogenicity. The biological significance of this activation for viroid symptom expression remains to be understood.

Recent studies on PSTVd and PLMVd have started to shed light on the molecular mechanisms underlying pathogenicity. A U257A change in the CCR converted several strains of PSTVd into lethal strains that caused severe growth stunting and premature death of infected plants (see **Figure 5**). The U257A substitution did not alter PSTVd secondary structure, replication levels, or tissue tropism of PSTVd. The stunted growth of infected tomato plants resulted from restricted cell growth, but not cell division or differentiation. This is correlated positively with downregulated expression of an expansin gene, *LeExp2*, that is known to play an important role in cell expansion in young growing organs. The peach calico symptom, characterized by extreme chlorosis of infected tissues, is associated with the insertion of an extra 12–13 nt sequence that folds into a hairpin in the left-terminal loop of PLMVd. Intriguingly, the insertion occurs sporadically *de novo* and can be acquired or lost during infection. Recent work shows that presence of this hairpin impairs processing and accumulation of chloroplast rRNAs. This eventually affects the structure and function of the chloroplast translation machinery. Chloroplast development is severely disturbed. Still, the underdeveloped chloroplasts retain the capacity to import proteins encoded by nuclear genes, which includes a chloroplast RNA polymerase, and support PLMVd replication. Altogether, these findings support the view that specific viroid sequence/structural elements can interact with yet to be identified host factors in a highly specific manner to alter host gene expression and developmental processes.

An emerging model for viroid pathogenicity is that small RNAs of 20–24 nt derived from viroid RNA sequences during infection can guide RNA silencing of host genes, thereby leading to development of disease symptoms. Consistent with this hypothesis, there is a positive correlation between the levels of small RNAs and symptom severity for PSTVd and ASBVd. Moreover, symptom development is correlated with production of small RNAs in some transgenic tomato lines expressing nonreplicating, hairpin PSTVd RNAs.

However, the correlation between viroid small RNA accumulation and symptom expression is not universal. It also remains to be tested whether viroid small RNAs can indeed target host genes for silencing and whether such silencing is crucial for disease development.

Virusoids

Virusoid, a term used less frequently today, refers to a group of circular satellite RNAs associated with viruses in the genus Sobemovirus, the members of which have nonenveloped icosahedral virions containing one molecule of linear, single-stranded and positive-sense RNA. They bear structural similarity with viroids but share biological properties with satellites. Satellite RNAs are found to be mostly associated with plant viruses. All satellite RNAs must coinfect with a helper virus in order to replicate. The satellite RNAs have little sequence similarity with their helper viruses. They replicate on their own templates by utilizing the replicating enzymes encoded by the helper viruses.

The virusoid RNA genomes are 220–388 nt long. They have a single-stranded, circular genome assuming rod-shaped secondary structure due to intramolecular base pairing. A virusoid genome does not code for any proteins. In these aspects, virusoids are similar to viroids. However, their thermostability is distinct from that of viroids, showing no cooperativity but rather random base sequences. Furthermore, like satellite RNAs, virusoids are replicated by the helper virus RNA-dependent RNA polymerases in the cytoplasm and are encapsidated by the coat proteins encoded by the helper viruses. They are encapsidated separately from the helper viral RNAs.

Five virusoids are currently known (**Table 3**). All helper viruses are members of the Sobemovirus family. These include *Rice yellow mottle virus* (RYMV), *Lucerne transient streak virus* (LTSV), *Subterranean clover mottle virus*, *Velvet tobacco mottle virus*, and *Solanum nodiflorum mottle virus*. By convention, the encapsidated and infectious form of the RNA is designated as the (+)-strand. It accumulates to higher levels than the (–)-strand that is produced during RNA–RNA transcription. Virusoids replicate via rolling circle mechanisms similar to viroids. Both the (+)- and (–)-strands of virusoid vLTSV contain hammerhead ribozyme activity *in vitro*, which catalyzes self-cleavage during replication.

There is little understanding of the biology of virusoids, in terms of their interactions with helper viruses, how they initiate replication, how they move between cells and through a plant, and how they influence viral disease symptoms. A recent study revealed no involvement of virusoid vRYMV in symptom modulation or ability to break host–plant resistance to the viral disease.

Conclusion

Viroids and virusoids are RNAs that are small in size, simple in structure, and yet complicated in biological functions. Their replication and systemic trafficking raise the fascinating questions of what RNA structural motifs within the RNA direct all of the biological functions and what host factors are employed by these motifs to accomplish each function necessary to establish a systemic infection. The viroid disease can be considered as an example of RNA-regulated expression of host genes. Further studies on viroid–host interactions and virusoid–host–helper virus interactions are expected to contribute new and exciting knowledge about the evolution of RNA-based pathogens and about the basic mechanisms of noncoding RNA functions. As compared to viroids, the biology of virusoids has been greatly understudied. It can be anticipated that with appropriate experimental tools developed, virusoids can serve as another powerful set of simple RNA models, like viroids, for fundamental discoveries in biology.

Table 3 Virusoids

Virusoid	*Helper virus*	*Genome size (nt)*	*Accession #*	*(+)-strand ribozyme*	*(–)-strand ribozyme*
vRYMV	RYMV	220	AF039909	HH	HH
vLSTV	LSTV	324	X01984	HH	
vSCMoV	SCMoV	332	M33000	HH	
		388	M33001		
vVTMoV	VTMoV	366	J02439	HH	
vSNMV	SNMV	377	J02388	HH	

RYMV, *Rice yellow mottle virus*; LSTV, *Lucerne transient streak virus*; SCMoV, *subterranean clover mottle virus*; VTMoV, *Velvet tobacco mottle virus*, SNMV, *Solanum nodiflorum mottle virus*; HH, Hammerhead.

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Relevant Website

http://subviral.med.uottawa.ca/cgi-bin/home.cgi – Subviral RNA Database

Yeasts

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Defining Statement
Definition and Classification of Yeasts
Yeast Ecology
Yeast Cell Structure
Nutrition, Metabolism, and Growth of Yeasts
Yeast Genetics
Industrial, Agricultural, and Medical Importance of Yeasts
Further Reading

Glossary

bioethanol Ethyl alcohol produced by yeast fermentation for use as a renewable biofuel.

birth scar Concave indentations that remain on the surface of daughter cells following budding.

budding A mode of vegetative reproduction in many yeast species in which a small outgrowth, the daughter bud, grows from the surface of a mother cell and eventually separates to form a new cell during cell division.

bud scar The chitin-rich, convex, ringed protrusions that remain on the mother cell surface of budding yeasts following the birth of daughter cells.

Candida albicans Common opportunistic human pathogenic yeast causing candidosis.

Crabtree effect The suppression of yeast respiration by high levels of glucose. This phenomenon is found in *Saccharomyces cerevisiae* cells, which continue to ferment irrespective of oxygen availability due to glucose repressing or inactivating the respiratory enzymes or due to the inherent limited capacity of cells to respire.

fission A mode of vegetative reproduction found in the yeast genus *Schizosaccharomyces*. Fission yeasts grow lengthwise and divide by forming a cell septum that constricts mother cells into two equal-sized daughters.

Pasteur effect Under anaerobic conditions, glycolysis proceeds faster than it does under aerobic conditions. In *Saccharomyces cerevisiae*, the Pasteur effect is observable only when the glucose concentration is low ($<\sim$5 mM) or in nutrient-deficient cells.

respirofermentation Fermentative metabolism of yeast in the presence of oxygen.

Saccharomyces cerevisiae Baker's or brewer's yeast species, which is used widely in the food and fermentation industries and is also being exploited in modern biotechnology (e.g., in the production of recombinant proteins) and as a model eukaryotic cell in fundamental biological research.

sporulation The production of haploid spores when sexually reproductive yeasts conjugate and undergo meiosis.

Abbreviations

AFLP	amplified fragment length polymorphism
AFM	Atomic force microscopy
CDI	cyclin-dependent kinase inhibitor
DEAE	diethylaminoethyl
ER	endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
GAP	general amino acid permease
NAD	nicotinamide adenine dinucleotide
RAPD	random amplified polymorphic DNA
YEPG	yeast extract peptone glucose
YNB	yeast nitrogen base

Defining Statement

Yeasts are eukaryotic unicellular microfungi that play important roles in industry, the environment, and medical science. This article describes the classification, ecology, cytology, metabolism, and genetics of yeast, with specific reference to *Saccharomyces cerevisiae* – baker's yeast. The biotechnological potential of yeasts, including their exploitation in food, fermentation, and pharmaceutical industries, is also discussed in the article.

Definition and Classification of Yeasts

Definition and Characterization of Yeasts

Yeasts are recognized as unicellular fungi that reproduce primarily by budding, and occasionally by fission, and that do not form their sexual states (spores) in or on a fruiting body. Yeast species may be identified and characterized according to various criteria based on cell morphology (e.g., mode of cell division and spore shape), physiology (e.g., sugar fermentation tests), immunology (e.g., immunofluorescence), and molecular biology (e.g., ribosomal DNA phylogeny, DNA reassociation, DNA base composition and hybridization, karyotyping, random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) of D1/D2 domain sequences of 26S rDNA). Molecular sequence analyses are being increasingly used by yeast taxonomists to categorize new species.

Yeast Taxonomy

The most commercially exploited yeast species, *S. cerevisiae* (baker's yeast), belongs to the fungal kingdom subdivision Ascomycotina. **Table 1** summarizes the taxonomic hierarchy of yeasts, with *S. cerevisiae* as an example.

Other yeast genera are categorized under Basidiomycotina (e.g., *Cryptococcus* spp. and *Rhodotorula* spp.) and Deuteromycotina (e.g., *Candida* spp. and *Brettanomyces* spp.). There are around 100 recognized yeast genera and the reader is directed to Kurtzman and Fell (1998) for additional information on yeast taxonomy.

Yeast Biodiversity

Around 1000 species of yeast have been described, but new species are being characterized on a regular basis and there is considerable untapped yeast biodiversity on Earth. For example, it has been estimated (in 1996) that only 0.065% of yeast genera (total 62 000) and 0.22% of yeast species (total 669 000) have been isolated and characterized. This means that there is an immense gap in our knowledge regarding biodiversity and the available 'gene pool' of wild natural isolates of yeast. Several molecular biological techniques are used to assist in the detection of new yeast species in the natural environment, and together with input from cell physiologists, they provide ways to conserve and exploit yeast biodiversity. *S. cerevisiae* is the most studied and exploited of all the yeasts, but the biotechnological potential of non-*Saccharomyces* yeasts is gradually being realized, particularly with regard to recombinant DNA technology (see **Table 8**).

Table 1 Taxonomic hierarchy of yeast

Taxonomic category	*Example (*Saccharomyces cerevisiae*)*
Kingdom	Fungi
Division	Ascomycota
Subdivision	Ascomycotina
Class	Hemiascomycete
Order	Endomycetales
Family	Saccharomycetacae
Subfamily	Saccharomyetoideae
Genus	*Saccharomyces*
Species	*cerevisiae*

Yeast Ecology

Natural Habitats of Yeast Communities

Yeasts are not as ubiquitous as bacteria in the natural environment, but nevertheless they can be isolated from soil, water, plants, animals, and insects. Preferred yeast habitats are plant tissues (leaves, flowers, and fruits), but a few species are found in commensal or parasitic relationships with animals. Some yeasts, most notably *Candida albicans*, are opportunistic human pathogens. Several species of yeast may be isolated from specialized or extreme environments, such as those with low water potential (i.e., high sugar or salt concentrations), low temperature (e.g., some psychrophilic yeasts have been isolated from polar regions), and low oxygen availability (e.g., intestinal tracts of animals). **Table 2** summarizes the main yeast habitats.

Yeasts in the Food Chain

Yeasts play important roles in the food chain. Numerous insect species, notably *Drosophila* spp., feed on yeasts that colonize plant material. As insect foods, ascomycetous yeasts convert low-molecular-weight nitrogenous compounds into proteins beneficial to insect nutrition. In addition to providing a food source, yeasts may also affect the physiology and sexual reproduction of drosophilids. In marine environments, yeasts may serve as food for filter feeders.

Microbial Ecology of Yeasts

In microbial ecology, yeasts are not involved in biogeochemical cycling as much as bacteria or filamentous fungi. Nevertheless, yeasts can use a wide range of carbon sources and thus play an important role as saprophytes in the carbon cycle, degrading plant detritus to carbon dioxide. In the cycling of nitrogen, some yeasts can reduce nitrate or ammonify nitrite, although most yeasts assimilate ammonium ions or amino acids into organic nitrogen. Most yeasts can reduce sulfate, although some are sulfur auxotrophs.

Table 2 Natural yeast habitats

Habitat	*Comments*
Soil	Soil may only be a reservoir for the long-term survival of many yeasts, rather than a habitat for growth. However, yeasts are ubiquitous in cultivated soils (about 10 000 yeast cells per gram of soil) and are found only in the upper, aerobic soil layers (10–15 cm). Some genera are isolated exclusively from soil (e.g., *Lipomyces* and *Schwanniomyces*)
Water	Yeasts predominate in surface layers of fresh and salt waters, but are not present in great numbers (about 1000 cells per liter). Many aquatic yeast isolates belong to red pigmented genera (*Rhodotorula*). *Debaryomyces hansenii* is a halotolerant yeast that can grow in nearly saturated brine solutions
Atmosphere	A few viable yeast cells may be expected per cubic meter of air. From layers above soil surfaces, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, and *Debaryomyces* spp. are dispersed by air currents
Plants	The interface between soluble nutrients of plants (sugars) and the septic world are common niches for yeasts (e.g., the surface of grapes); the spread of yeasts on the phyllosphere is aided by insects (e.g., *Drosophila* spp.); a few yeasts are plant pathogens. The presence of many organic compounds on the surface and decomposing areas (exudates, flowers, fruits, phyllosphere, rhizosphere, and necrotic zones) creates conditions favorable for growth of many yeasts
Animals	Several nonpathogenic yeasts are associated with the intestinal tract and skin of warm-blooded animals; several yeasts (e.g., *Candida albicans*) are opportunistically pathogenic toward humans and animals; numerous yeasts are commensally associated with insects, which act as important vectors in the natural distribution of yeasts
Built environment	Yeasts are fairly ubiquitous in buildings, for example, *Aureobasidium pullulans* (black yeast) is common on damp household wallpaper and *Saccharomyces cerevisiae* is readily isolated from surfaces (pipework and vessels) in wineries

Yeast Cell Structure

General Cellular Characteristics

Yeasts are unicellular eukaryotes that have ultrastructural features similar to that of higher eukaryotic cells. This, together with their ease of growth, and amenability to biochemical, genetic, and molecular biological analyses, makes yeasts model organisms in studies of eukaryotic cell biology. Yeast cell size can vary widely, depending on the species and conditions of growth. Some yeasts may be only 2–3 μm in length, whereas others may attain lengths of 20–50 μm. Cell width appears less variable, between 1 and 10 μm. *S. cerevisiae* is generally ellipsoid in shape with a large diameter of 5–10 μm and a small diameter of 1–7 μm. **Table 3** summarizes the diversity of yeast cell shapes.

Table 3 Diversity of yeast cell shapes

Cell shape	*Description*	*Examples of yeast genera*
Ellipsoid	Ovoid-shaped cells	*Saccharomyces*
Cylindrical	Elongated cells with hemispherical ends	*Schizosaccharomyces*
Apiculate	Lemon shaped	*Hanseniaspora*, *Saccharomycodes*
Ogival	Elongated cell rounded at one end and pointed at other	*Dekkera*, *Brettanomyces*
Flask shaped	Cells dividing by bud fission	*Pityrosporum*
Pseudohyphal	Chains of budding yeast cells, which have elongated without detachment. Pseudohyphal morphology is intermediate between a chain of yeast cells and a hypha	Occasionally found in starved cells of *Saccharomyces cerevisiae* and frequently in *Candida albicans* (filamentous cells form from 'germ tubes', and hyphae may give rise to buds called blastospores)
Hyphal	Basidiomycetous yeast cells grow lengthwise to form branched or unbranched threads or true hyphae, occasionally with septa (cross walls) to make up mycelia. Septa may be laid down by the continuously extending hyphal tip	*Saccharomycopsis* spp.
Dimorphic	Yeasts that grow vegetatively in either yeast or filamentous forms	*C. albicans*, *Saccharomycopsis fibuligera*, *Kluyveromyces marxianus*, *Malassezia furfur*, *Yarrowia lipolytica*, *Ophiostoma novo-ulmi*, *Sporothrix schenkii*, *Histoplasma capsulatum*
Miscellaneous		
	Triangular	*Trigonopsis*
	Curved	*Cryptococcus*
	Stalked	*Sterigmatomyces*
	Spherical	*Debaryomyces*

Several yeast species are pigmented and various colors may be visualized in surface-grown colonies, for example, cream (e.g., *S. cerevisiae*), white (e.g., *Geotrichum* spp.), black (e.g., *Aureobasidium pullulans*), pink (e.g., *Phaffia rhodozyma*), red (e.g., *Rhodotorula* spp.), orange (e.g., *Rhodosporidium* spp.), and yellow (e.g., *Bullera* spp.). Some pigmented yeasts have applications in biotechnology. For example, the astaxanthin pigments of *P. rhodozyma* have applications as fish feed colorants for farmed salmonids, which have no means of synthesizing these red compounds.

Methods in Yeast Cytology

By using various cytochemical and cytofluorescent dyes and phase contrast microscopy, it is possible to visualize several subcellular structures in yeasts (e.g., cell walls, capsules, nuclei, vacuoles, mitochondria, and several cytoplasmic inclusion bodies). The *GFP* gene from the jellyfish (*Aequorea victoria*) encodes the green fluorescent protein (which fluoresces in blue light) and can be used to follow the subcellular destiny of certain expressed proteins when GFP is fused with the genes of interest. Immunofluorescence can also be used to visualize yeast cellular features when dyes such as fluorescein isothiocyanate and rhodamine B are conjugated with monospecific antibodies raised against yeast structural proteins. Confocal scanning laser immunofluorescence microscopy can also be used to detect the intracellular localization of proteins within yeast cells and to give three-dimensional ultrastructural information. Fluorescence-activated cell sorting (FACS) has proven very useful in studies of the yeast cell cycle and in monitoring changes in organelle (e.g., mitochondrial) biogenesis. Scanning electron microscopy is useful in revealing the cell surface topology of yeasts, as is atomic force microscopy, which has achieved high-contrast nanometer resolution for yeast cell surfaces (**Figure 1**). Transmission electron microscopy, however, is essential for visualizing the intracellular fine structure of ultrathin yeast cell sections (**Figure 2**).

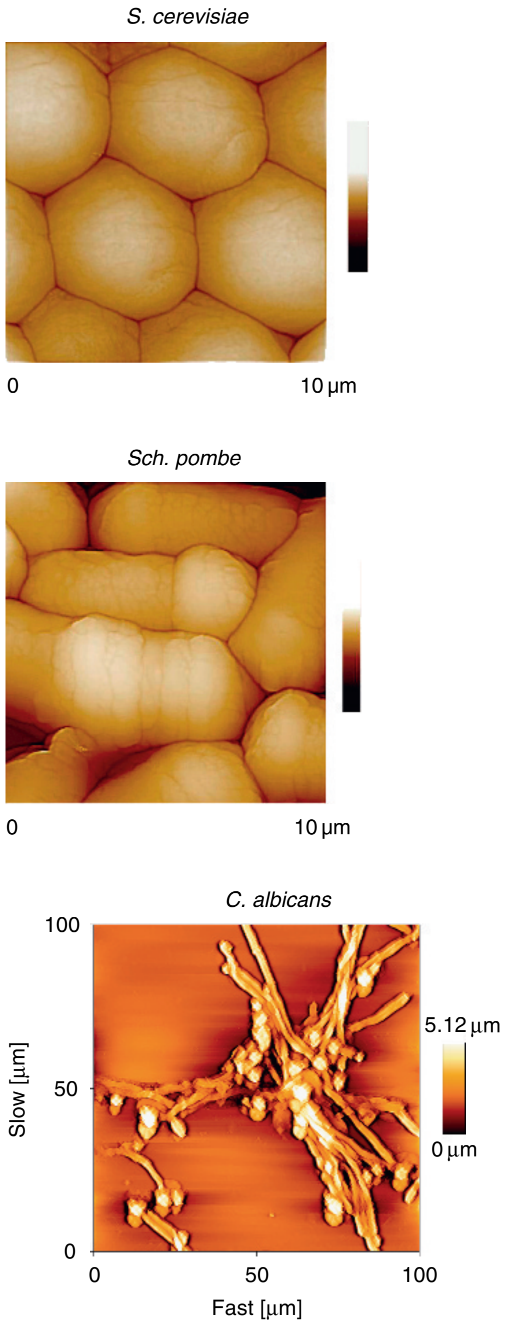

Figure 1 Atomic force microscopy (AFM) of yeast cell surfaces. Courtesy of Dr. A Adya and Dr. E Canetta, University of Abertay Dundee.

Subcellular Yeast Architecture and Function

Transmission electron microscopy of a yeast cell typically reveals the cell wall, nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, microbodies, and secretory vesicles. **Figure 2** shows an electron micrograph of a typical yeast cell.

Several of these organelles are not completely independent of each other and derive from an extended intramembranous system. For example, the movement and positioning of organelles depends on the cytoskeleton, and the trafficking of proteins in and out of cells relies on vesicular communication between the ER, Golgi apparatus, vacuole, and plasma membrane. Yeast organelles can be readily isolated for further studies by physical, chemical, or enzymatic disruption of the cell wall, and the purity of organelle preparations can be evaluated using specific marker enzyme assays.

In the yeast cytoplasm, ribosomes and occasionally plasmids (e.g., 2 µm circles) are found, and the structural

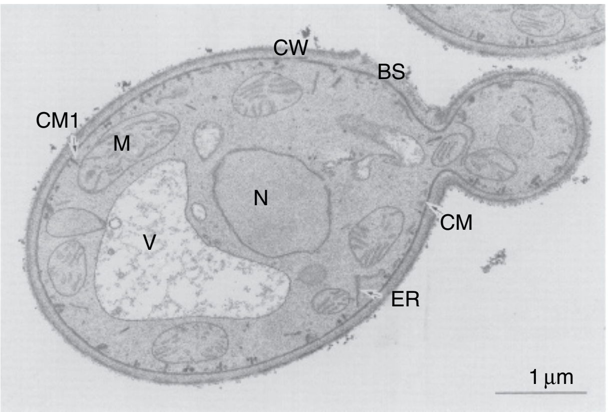

Figure 2 Ultrastructural features of a yeast cell. The transmission electron micrograph is of a *Candida albicans* cell. BS, bud scar; CM, cell membrane; CMI, cell membrane invagination; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; and V, vacuole. Courtesy of M Osumi, Japan Women's University, Tokyo.

organization of the cell is maintained by a cytoskeleton of microtubules and actin microfilaments. The yeast cell envelope, which encases the cytoplasm, comprises (from the inside looking out) the plasma membrane, periplasm, cell wall, and, in certain yeasts, a capsule and a fibrillar layer. Spores encased in an ascus may be revealed in those yeasts that undergo differentiation following sexual conjugation and meiosis. **Table 4** provides a summary of the physiological functions of the various structural components found in yeast cells.

Nutrition, Metabolism, and Growth of Yeasts

Nutritional and Physical Requirements for Yeast Growth

Yeast nutritional requirements

Yeast cells require macronutrients (sources of carbon, nitrogen, oxygen, sulfur, phosphorus, potassium, and magnesium) at the millimolar level in growth media, and they require trace elements (e.g., Ca, Cu, Fe, Mn, and Zn) at the micromolar level. Most yeasts grow quite well in simple nutritional media, which supply carbon–nitrogen backbone compounds together with inorganic ions and a few growth factors. Growth factors are organic compounds required in very low concentrations for specific catalytic or structural roles in yeast, but are not used as energy sources. Yeast growth factors include vitamins, which serve vital functions as components of coenzymes; purines and pyrimidines; nucleosides and nucleotides; amino acids; fatty acids; sterols; and other miscellaneous compounds (e.g., polyamines and choline). Growth factor requirements vary among yeasts, but when a yeast species is said to have a growth factor requirement, it indicates that the species cannot synthesize the particular factor, resulting in the curtailment of growth without its addition to the culture medium.

Yeast culture media

It is quite easy to grow yeasts in the laboratory on a variety of complex and synthetic media. Malt extract or yeast extract supplemented with peptone and glucose (as

Table 4 Functional components of an ideal yeast cell

Organelle or cellular structure	*Function*
Cell envelope	Comprises the plasma membrane that acts as a selectively permeable barrier for transport of hydrophilic molecules in and out of fungal cells; the periplasm containing proteins and enzymes unable to permeate the cell wall; the cell wall that provides protection and shape and is involved in cell–cell interactions, signal reception, and specialized enzyme activities; fimbriae involved in sexual conjugation; and capsules to protect cells from dehydration and immune cell attack
Nucleus	Contains chromosomes (DNA–protein complexes) that pass genetic information to daughter cells during cell division and the nucleolus, which is the site of ribosomal RNA transcription and processing
Mitochondria	Responsible, under aerobic conditions, for respiratory metabolism and, under anaerobic conditions, for fatty acid, sterol, and amino acid metabolism
Endoplasmic reticulum	Ribosomes on the rough endoplasmic reticulum are the sites of protein biosyntheses (translation of mRNA nucleotide sequences into amino acid sequences in a polypeptide chain)
Proteasome	Multi-subunit protease complexes involved in regulating protein turnover
Golgi apparatus and vesicles	Secretory system for import (endocytosis) and export (exocytosis) of proteins
Vacuole	Intracellular reservoir (amino acids, polyphosphate, and metal ions), proteolysis, protein trafficking, and control of intracellular pH
Peroxisome	Present in some methylotrophic (methanol-utilizing) yeasts for oxidative utilization of specific carbon and nitrogen sources (contain catalase and oxidases). Glyoxysomes contain enzymes of the glyoxylate cycle

Reproduced from Walker GM and White NA (2005) Introduction to fungal physiology. In: Kavanagh K (ed.) *Fungi: Biology and Applications*, ch. 2, pp. 1–34. Chichester, UK: John Wiley & Sons.

in YEPG) is commonly employed for the maintenance and growth of most yeasts. Yeast nitrogen base (YNB) is a commercially, available chemically defined medium that contains ammonium sulfate and asparagine as nitrogen sources, together with mineral salts, vitamins, and trace elements. The carbon source of choice (e.g., glucose) is usually added to a final concentration of 1% (w/v). For the continuous cultivation of yeasts in chemostats, media that ensure that all the nutrients for growth are present in excess except one (the growth-limiting nutrient) are usually designed. Chemostats can therefore facilitate studies on the influence of a single nutrient (e.g., glucose, in carbon-limited chemostats) on yeast cell physiology, with all other factors being kept constant. In industry, yeasts are grown in a variety of fermentation feedstocks, including malt wort, molasses, grape juice, cheese whey, glucose syrups, and sulfite liquor.

Physical requirements for yeast growth

Most yeast species thrive in warm, dilute, sugary, acidic, and aerobic environments. Most laboratory and industrial yeasts (e.g., *S. cerevisiae* strains) grow best from 20 to 30 °C. The lowest maximum temperature for growth of yeasts is around 20 °C, whereas the highest is around 50 °C.

Yeasts need water in high concentration for growth and metabolism. Several food spoilage yeasts (e.g., *Zygosaccharomyces* spp.) are able to withstand conditions of low water potential (i.e., high sugar or salt concentrations), and such yeasts are referred to as osmotolerant or xerotolerant.

Most yeasts grow very well between pH 4.5 and 6.5. Media acidified with organic acids (e.g., acetic and lactic) are more inhibitory to yeast growth than are media acidified with mineral acids (e.g., hydrochloric). This is because undissociated organic acids can lower intracellular pH following their translocation across the yeast cell membrane. This forms the basis of the action of weak acid preservatives in inhibiting food spoilage yeast growth. Actively growing yeasts acidify their growth environment through a combination of differential ion uptake, proton secretion during nutrient transport (see later), direct secretion of organic acids (e.g., succinate and acetate), and carbon dioxide evolution and dissolution. Intracellular pH is regulated within relatively narrow ranges in growing yeast cells (e.g., around pH 5 in *S. cerevisiae*), mainly through the action of the plasma membrane proton-pumping ATPase.

Most yeasts are aerobes. Yeasts are generally unable to grow well under completely anaerobic conditions because, in addition to providing the terminal electron acceptor in respiration, oxygen is needed as a growth factor for membrane fatty acid (e.g., oleic acid) and sterol (e.g., ergosterol) biosynthesis. In fact, *S. cerevisiae* is auxotrophic for oleic acid and ergosterol under anaerobic conditions and this yeast is not, strictly speaking, a facultative anaerobe. **Table 5** categorizes yeasts based on their fermentative properties and growth responses to oxygen availability.

Carbon Metabolism by Yeasts

Carbon sources for yeast growth

As chemorganotrophic organisms, yeasts obtain carbon and energy in the form of organic compounds. Sugars are widely used by yeasts. *S. cerevisiae* can grow well on glucose, fructose, mannose, galactose, sucrose, and maltose. These sugars are also readily fermented into ethanol and carbon dioxide by *S. cerevisiae*, but other carbon substrates such as ethanol, glycerol, and acetate can be respired by *S. cerevisiae* only in the presence of oxygen. Some yeasts (e.g., *Pichia stipitis* and *Candida shehatae*) can use five-carbon pentose sugars such as D-xylose and L-arabinose as growth and fermentation substrates. A few amylolytic yeasts (e.g., *Saccharomyces diastaticus* and *Schwanniomyces occidentalis*) that can use starch exist, and several oleaginous yeasts (e.g., *Candida tropicalis* and *Yarrowia lipolytica*) can grow on hydrocarbons, such as straight-chain alkanes in the C_{10}–C_{20} range. Several methylotrophic yeasts (e.g., *Hansenula polymorpha* and *Pichia pastoris*) can grow very well on methanol as the sole carbon and energy source, and these yeasts have

Table 5 Classification of yeasts based on fermentative property/growth response to oxygen availability

Class	*Examples*	*Comments*
Obligately fermentative	*Candida pintolopesii (Saccharomyces telluris)*	Naturally occurring respiratory-deficient yeasts. Only ferment, even in the presence of oxygen
Facultatively fermentative		
Crabtree-positive	*Saccharomyces cerevisiae*	Such yeasts predominantly ferment high-sugar-containing media in the presence of oxygen (respirofermentation)
Crabtree-negative	*Candida utilis*	Such yeasts do not form ethanol under aerobic conditions and cannot grow anaerobically
Nonfermentative	*Rhodotorula rubra*	Such yeasts do not produce ethanol, in either the presence or absence of oxygen

industrial potential in the production of recombinant proteins using methanol-utilizing genes as promoters.

Yeast sugar transport

Sugars are transported into yeast cells across the plasma membrane by various mechanisms such as simple net diffusion (a passive or free mechanism), facilitated (catalyzed) diffusion, and active (energy-dependent) transport. The precise mode of sugar translocation will depend on the sugar, yeast species, and growth conditions. For example, *S. cerevisiae* takes up glucose by facilitated diffusion and maltose by active transport. Active transport means that the plasma membrane ATPases act as directional proton pumps in accordance with chemiosmotic principles. The pH gradients thus drive nutrient transport either via proton symporters (as is the case with certain sugars and amino acids) or via proton antiporters (as is the case with potassium ions).

Yeast sugar metabolism

The principal metabolic fates of sugars in yeasts are the dissimilatory pathways of fermentation and respiration (shown in **Figure 3**) and the assimilatory pathways of gluconeogenesis and carbohydrate biosynthesis. Yeasts described as fermentative are able to use organic substrates (sugars) anaerobically as electron donors, electron acceptors, and carbon sources. During alcoholic fermentation of sugars, *S. cerevisiae* and other fermentative yeasts reoxidize the reduced coenzyme NADH to NAD (nicotinamide adenine dinucleotide) in terminal step reactions from pyruvate. In the first of these terminal reactions, catalyzed by pyruvate decarboxylase, pyruvate is decarboxylated to acetaldehyde, which is finally reduced by alcohol dehydrogenase to ethanol. The regeneration of NAD is necessary to maintain the redox balance and prevent the stalling of glycolysis. In alcoholic beverage fermentations (e.g., of beer, wine, and distilled spirits), other fermentation metabolites, in addition to ethanol and carbon dioxide, that are very important in the development of flavor are produced by yeast. These metabolites include fusel alcohols (e.g., isoamyl alcohol), polyols (e.g., glycerol), esters (e.g., ethyl acetate), organic acids (e.g., succinate), vicinyl diketones (e.g., diacetyl), and aldehydes (e.g., acetaldehyde). The production of glycerol (an important industrial commodity) can be enhanced in yeast fermentations by the addition of sulfite, which chemically traps acetaldehyde.

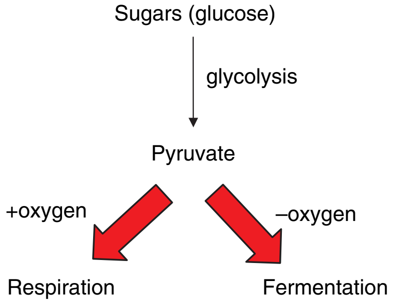

Figure 3 Overview of sugar catabolic pathways in yeast cells. Reproduced from Walker (1998) *Yeast Physiology and Biotechnology*. Chichester, UK: John Wiley & Sons Limited.

$$\text{Glucose} + \text{HSO}_3^- \rightarrow \text{glycerol} + \text{acetaldehyde}-\text{HSO}_3^- + \text{CO}_2$$

Aerobic respiration of glucose by yeasts is a major energy-yielding metabolic route and involves glycolysis, the citric acid cycle, the electron transport chain, and oxidative phosphorylation. The citric acid cycle (or Krebs cycle) represents the common pathway for the oxidation of sugars and other carbon sources in yeasts and filamentous fungi and results in the complete oxidation of one pyruvate molecule to $2CO_2$, 3NADH, $1FADH_2$, $4H^+$, and 1GTP.

Of the environmental factors that regulate respiration and fermentation in yeast cells, the availability of glucose and oxygen is best understood and is linked to the expression of regulatory phenomena, referred to as the Pasteur effect and the Crabtree effect. A summary of these phenomena is provided in **Table 6**.

Nitrogen Metabolism by Yeasts

Nitrogen sources for yeast growth

Although yeasts cannot fix molecular nitrogen, simple inorganic nitrogen sources such as ammonium salts are widely used. Ammonium sulfate is a commonly used nutrient in yeast growth media because it provides a source of both assimilable nitrogen and sulfur. Some yeasts can also grow on nitrate as a source of nitrogen, and, if able to do so, may also use subtoxic concentrations of nitrite. A variety of organic nitrogen compounds (amino acids, peptides, purines, pyrimidines, and amines) can also provide the nitrogenous requirements of the yeast cell. Glutamine and aspartic acids are readily deaminated by yeasts and therefore act as good nitrogen sources.

Yeast transport of nitrogenous compounds

Ammonium ions are transported in *S. cerevisiae* by both high-affinity and low-affinity carrier-mediated transport systems. Two classes of amino acid uptake systems operate in yeast cells. One is broadly specific, the general amino acid permease (GAP), and effects the uptake of all naturally occurring amino acids. The other system includes a variety of transporters that display specificity for one or a small number of related amino acids. Both the general and the specific transport systems are energy dependent.

Table 6 Summary of regulatory phenomena in yeast sugar metabolism

Phenomenon	*Description*	*Examples of yeasts*
Pasteur effect	Activation of sugar metabolism by anaerobiosis	*Saccharomyces cerevisiae* (resting or starved cells)
Crabtree effect (short-term)	Rapid ethanol production in aerobic conditions due to sudden excess of glucose (that acts to inactivate respiratory enzymes)	*S. cerevisiae* and *Schizosaccharomyces pombe*
Crabtree effect (long-term)	Ethanol production in aerobic conditions when excess glucose acts to repress respiratory genes	*S. cerevisiae* and *Sch. pombe*
Custers effect	Stimulation of ethanol fermentation by oxygen	*Dekkera* and *Brettanomyces* spp.
Kluyver effect	Anaerobic fermentation of glucose, but not of certain other sugars (disaccharides)	*Candida utilis*

Yeast metabolism of nitrogenous compounds

Yeasts can incorporate either ammonium ions or amino acids into cellular protein, or these nitrogen sources can be intracellularly catabolized to serve as nitrogen sources. Yeasts also store relatively large pools of endogenous amino acids in the vacuole, most notably arginine. Ammonium ions can be directly assimilated into glutamate and glutamine, which serve as precursors for the biosynthesis of other amino acids. The precise mode of ammonium assimilation adopted by yeasts will depend mainly on the concentration of available ammonium ions and the intracellular amino acid pools. Amino acids may be dissimilated (by decarboxylation, transamination, or fermentation) to yield ammonium and glutamate, or they may be directly assimilated into proteins.

Yeast Growth

The growth of yeasts is concerned with how cells transport and assimilate nutrients and then integrate numerous component functions in the cell in order to increase in mass and eventually divide. Yeasts have proven invaluable in unraveling the major control elements of the eukaryotic cell cycle, and research with the budding yeast, *S. cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, has significantly advanced our understanding of cell cycle regulation, which is particularly important in the field of human cancer. For example, two scientists, Leland Hartwell and Paul Nurse, were awarded the Nobel Prize for Medicine in 2002 for their pioneering studies on the control of cell division in budding and fission yeasts, respectively.

Vegetative reproduction in yeasts

Budding is the most common mode of vegetative reproduction in yeasts and is typical in ascomycetous yeasts such as *S. cerevisiae*. **Figure 4** shows a scanning electron micrograph of budding cells of *S. cerevisiae*. Yeast buds are initiated when mother cells attain a critical cell size at a time that coincides with the onset of DNA synthesis. This is followed by localized weakening of the cell wall and this, together with tension exerted by turgor pressure, allows the extrusion of the cytoplasm in an area bounded by the new cell wall material. The mother and daughter bud cell walls are contiguous during bud development. Multilateral budding is common in which daughter buds emanate from different locations on the mother cell surface. **Figure 5** shows multilateral budding in *S. cerevisiae*. In *S. cerevisiae*, cell size at division is asymmetrical, with buds being smaller than mother cells when they separate (**Figure 6**). Some yeast genera (e.g., *Hanseniaspora* and *Saccharomycodes*) undergo bipolar budding, where buds are restricted to the tips of lemon-shaped cells. Scar tissue on the yeast cell wall, known as the bud and birth scars, remain on the daughter bud and mother cells, respectively. These scars are rich in the polymer chitin and can be stained with fluorescent dyes (e.g., calcoflour white) to provide useful information regarding cellular age in *S. cerevisiae*, since the number of scars represents the number of completed cell division cycles.

Fission is a mode of vegetative reproduction typified by species of *Schizosaccharomyces*, which divide exclusively by forming a cell septum that constricts the cell into two equal-size daughters. In *Sch. pombe*, which has been used extensively in eukaryotic cell cycle studies, newly divided daughter cells grow lengthways in a monopolar fashion for about one-third of their new cell cycle. Cells then switch to bipolar growth for about three-quarters of the cell cycle until mitosis is initiated at a constant cell length stage.

Filamentous growth occurs in numerous yeast species and may be regarded as a mode of vegetative growth alternative to budding or fission. Some yeasts exhibit a propensity to grow with true hyphae initiated from germ tubes (e.g., *C. albicans*, **Figure 7**), but others (including *S. cerevisiae*) may grow in a pseudohyphal fashion when induced to do so by unfavorable conditions. Hyphal and pseudohyphal growth represent different developmental pathways in yeasts, but cells can revert to unicellular growth upon return to more conducive growth conditions. Filamentation may therefore represent an adaptation to foraging by yeasts when nutrients are scarce.

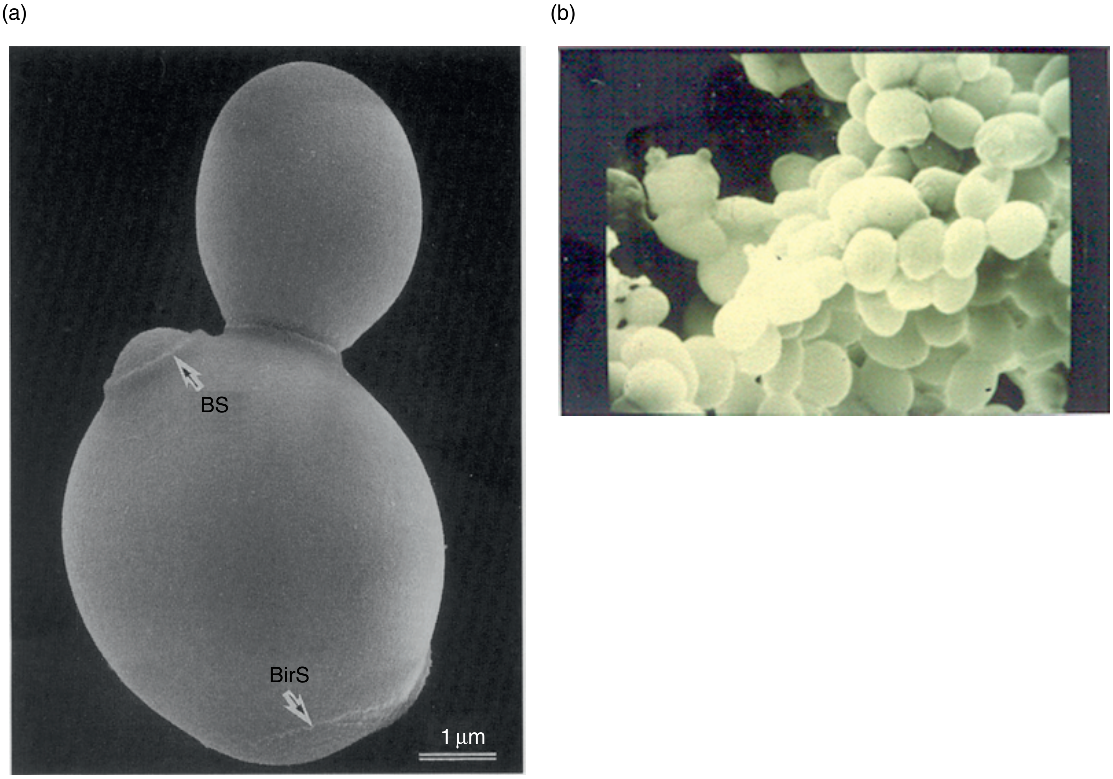

Figure 4 Scanning electron micrographs of budding yeast. (a) Individual cell. BS, bud scar; and BirS, birth scar. Courtesy of M Osumi, Japan Women's University: Tokyo. (b) Cluster of cells.

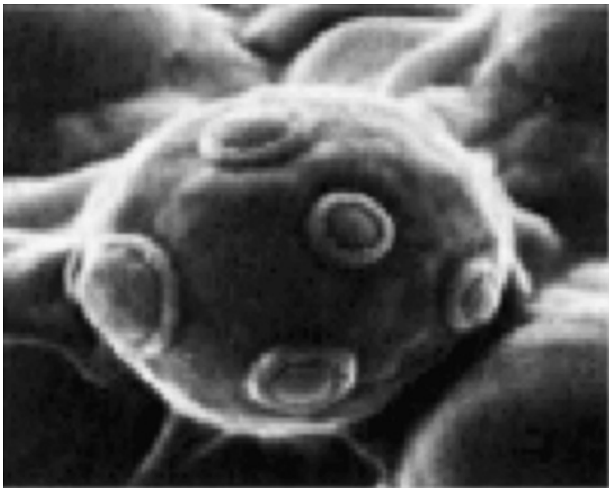

Figure 5 Bud scars in a single cell of *Saccharomyces cerevisiae*. The micrograph shows multilateral budding on the surface of an aged cell of *S. cerevisiae*. Courtesy of Prof. A Martini, University of Perugia, Italy.

Population growth of yeasts

As in most microorganisms, when yeast cells are inoculated into a liquid nutrient medium and incubated under optimal physical growth conditions, a typical batch growth curve will result when the viable cell population is plotted against time. This growth curve is made up of a lag phase (period of no growth, but physiological adaptation of cells to their new environment), an exponential phase (limited period of logarithmic cell doublings), and a stationary phase (resting period with zero growth rate).

Diauxic growth is characterized by two exponential phases and occurs when yeasts are exposed to two carbon growth substrates that are used sequentially. This occurs during aerobic growth of *S. cerevisiae* on glucose (the second substrate being ethanol formed from glucose fermentation).

In addition to batch cultivation of yeasts, cells can also be propagated in continuous culture in which exponential growth is prolonged without lag or stationary phases. Chemostats are continuous cultures that are based on the controlled feeding of a sole growth-limiting nutrient into an open culture vessel, which permits the outflow of cells and spent medium. The feeding rate is referred to as the dilution rate, which is employed to govern the yeast growth rate under the steady-state conditions that prevail in a chemostat.

Specialized yeast culture systems include immobilized bioreactors. Yeast cells can be readily immobilized or entrapped in a variety of natural and synthetic materials (e.g., calcium alginate gel, wood chips, hydroxyapatite ceramics, diethylaminoethyl (DEAE) cellulose, or microporous

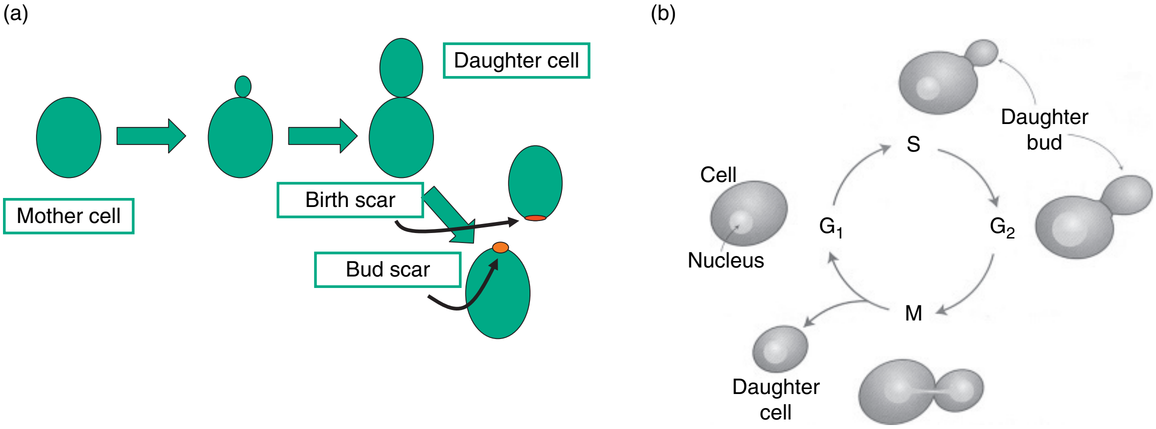

Figure 6 Budding processes in yeast. (a) Schematic diagram of budding. (b) Budding cell cycle, as typified by *Saccharomyces cerevisiae.* S, DNA synthesis period; G_1, pre-DNA synthesis gap period; G_2, post-DNA synthesis gap period; and M, mitosis. Reproduced from Madhani H (2007) *From a to α. Yeast as a Model for Cellular Differentiation*. New York: Cold Spring Harbor Laboratory Press.

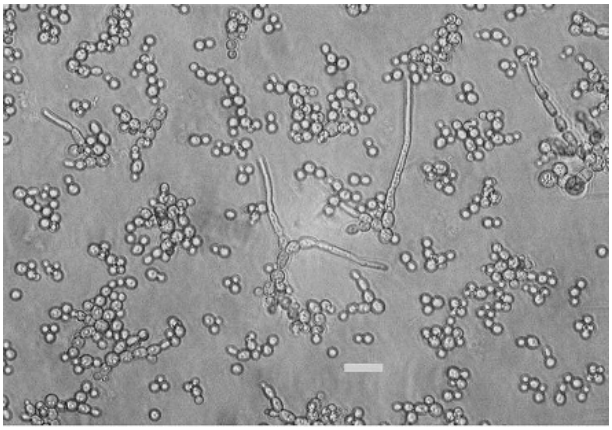

Figure 7 Dimorphism in *Candida albicans.* The micrograph shows a mixture of budding cells and hyphal forms of the yeast, which is an important human pathogen.

glass beads), and such materials have applications in the food and fermentation industries.

Yeast Genetics

Life Cycle of Yeasts

Many yeasts have the ability to reproduce sexually, but the processes involved are best understood in the budding yeast, *S. cerevisiae*, and the fission yeast, *Sch. pombe.* Both species have the ability to mate, undergo meiosis, and sporulate. The development of spores by yeasts represents a process of morphological, physiological, and biochemical differentiation of sexually reproductive cells.

Mating in *S. cerevisiae* involves the conjugation of two haploid cells of opposite mating types, designated as **a** and α (**Figure 8**). These cells synchronize one another's cell cycles in response to peptide mating pheromones, known as **a** factor and α factor.

The conjugation of mating cells occurs by cell wall surface contact followed by plasma membrane fusion to form a common cytoplasm. Karyogamy (nuclear fusion) then follows, resulting in a diploid nucleus. The stable diploid zygote continues the mitotic cell cycles in rich growth media, but if starved of nitrogen, the diploid cells sporulate to yield four haploid spores. These germinate in rich media to form haploid budding cells that can mate with each other to restore the diploid state. **Figure 9** shows mating and sporulation in *S. cerevisiae.*

In *Sch. pombe*, haploid cells of the opposite mating types (designated h^+ and h^-) secrete mating pheromones and, when starved of nitrogen, undergo conjugation to form diploids. In *Sch. pombe*, however, such diploidization is transient under starvation conditions and cells soon enter meiosis and sporulate to produce four haploid spores.

Genetic Manipulation of Yeasts

There are several ways of genetically manipulating yeast cells, including hybridization, mutation, rare mating, cytoduction, spheroplast fusion, single chromosome transfer, and transformation using recombinant DNA technology. Classic genetic approaches in *S. cerevisiae* involve mating of haploids of opposite mating types. Subsequent meiosis and sporulation result in the production of a *tetrad ascus* with four spores, which can be isolated, propagated, and genetically analyzed (i.e., tetrad analysis). This process forms the basis of genetic breeding programs for laboratory reference strains of *S. cerevisiae.* However, industrial (e.g., brewing) strains of this yeast are polyploid, are reticent to mate, and exhibit poor sporulation with low spore viability. It is, therefore, generally fruitless to perform tetrad analysis

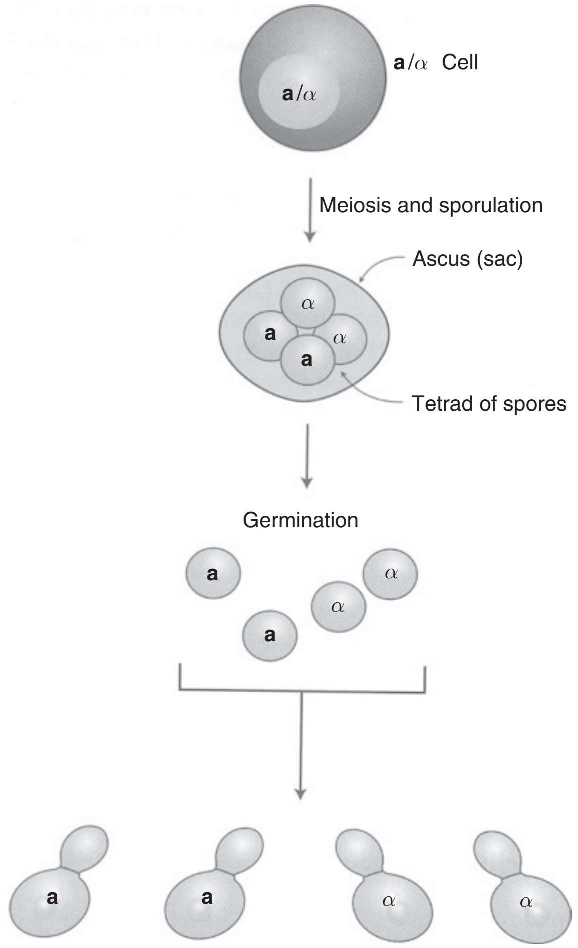

Figure 8 Sexual life cycle of *Saccharomyces cerevisiae*. Reproduced from Madhani H (2007). *From a to α. Yeast as a Model for Cellular Differentiation*. New York: Cold Spring Harbor Laboratory Press.

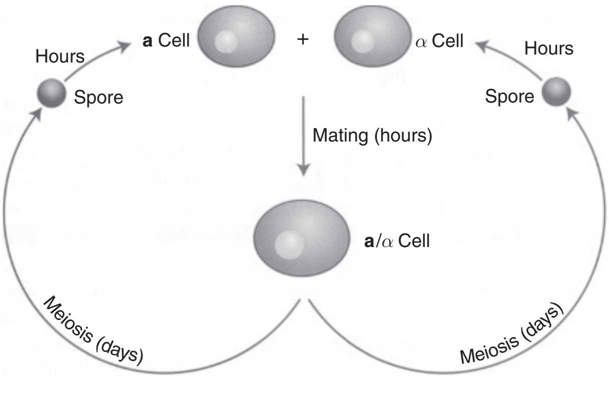

Figure 9 Meiosis and sporulation in *Saccharomyces cerevisiae*. Diploid (**a**/α) cells can undergo meiosis and sporulation to form spores that can germinate into **a** and α haploid cells. Reproduced from Madhani H (2007) *From a to α. Yeast as a Model for Cellular Differentiation*. New York: Cold Spring Harbor Laboratory Press.

and breeding with brewer's yeasts. Genetic manipulation strategies for preventing the sexual reproductive deficiencies associated with brewer's yeast include spheroplast fusion and recombinant DNA technology.

Intergeneric and intrageneric yeast hybrids may be obtained using the technique of spheroplast fusion. This involves the removal of yeast cell walls using lytic enzymes (e.g., glucanases from snail gut juice or microbial sources), followed by the fusion of the resulting spheroplasts in the presence of polyethylene glycol and calcium ions.

Recombinant DNA technology (genetic engineering) of yeast is summarized in **Figure 10** and transformation strategies in **Figure 11**. Yeast cells possess particular attributes for expressing foreign genes and have now become the preferred hosts, over bacteria, for producing certain human proteins for pharmaceutical use (e.g., insulin, human serum albumin, and hepatitis vaccine). Although the majority of research and development in recombinant protein synthesis in yeasts has been conducted using *S. cerevisiae*, several non-*Saccharomyces* species are being studied and exploited in biotechnology. For example, *H. polymorpha* and *P. pastoris* (both methylotrophic yeasts) exhibit particular advantages over *S. cerevisiae* in cloning technology (see **Table 8**).

Yeast Genome and Proteome Projects

A landmark in biotechnology was reached in 1996 with completion of the sequencing of the entire genome of *S. cerevisiae*. The *Sch. pombe* genome was sequenced in 2002. The functional analysis of the many orphan genes of *S. cerevisiae*, for which no function has yet been assigned, is under way through international research collaborations. Elucidation by cell physiologists of the biological function of all *S. cerevisiae* genes, that is, the complete analysis of the yeast proteome, will not only lead to an understanding of how a simple eukaryotic cell works, but also provide an insight into molecular biological aspects of heritable human disorders.

Industrial, Agricultural, and Medical Importance of Yeasts

Industrial Significance of Yeasts

Yeasts have been exploited for thousands of years in traditional fermentation processes to produce beer, wine, and bread. The products of modern yeast biotechnologies impinge on many commercially important sectors, including food, beverages, chemicals, industrial enzymes, pharmaceuticals, agriculture, and the environment (**Table 7**). *S. cerevisiae* represents the primary yeast 'cell factory' in biotechnology and is the most exploited

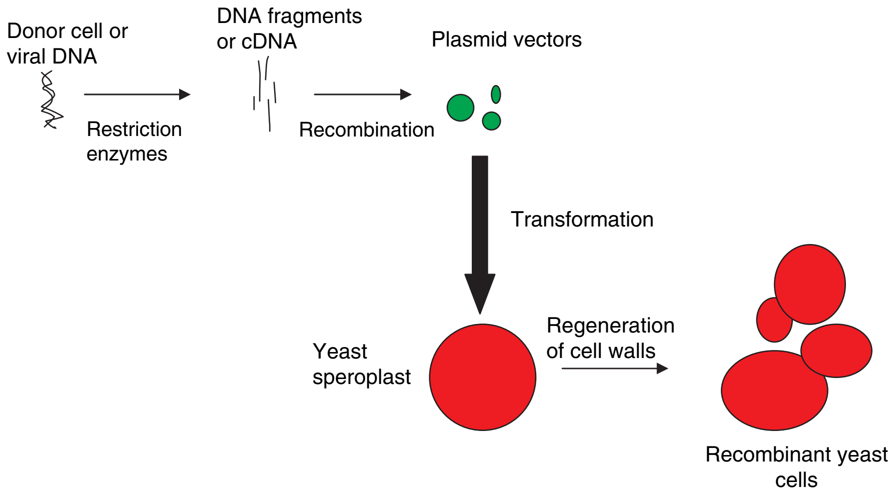
Donor cell or viral DNA
DNA fragments or cDNA
Plasmid vectors
Restriction enzymes
Recombination
Transformation
Yeast speroplast
Regeneration of cell walls
Recombinant yeast cells

Figure 10 Basic procedures in yeast genetic engineering. Reproduced from Walker GM (1998). *Yeast Physiology and Biotechnology*. Chichester, UK: John Wiley & Sons.

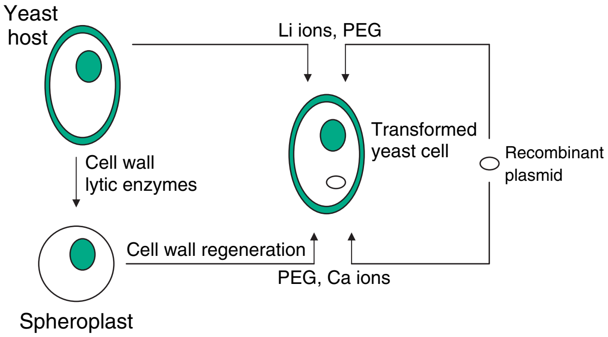
Yeast host
Li ions, PEG
Transformed yeast cell
Recombinant plasmid
Cell wall lytic enzymes
Cell wall regeneration
PEG, Ca ions
Spheroplast

Figure 11 Yeast transformation strategies. PEG, polyethylene glycol.

Table 7 Industrial commodities produced by yeasts

Commodity	*Examples*
Beverages	Potable alcoholic beverages: Beer, wine, cider, sake, and distilled spirits (whisky, rum, gin, vodka, and cognac)
Food and animal feed	Baker's yeast, yeast extracts, fodder yeast, livestock growth factor, and feed pigments
Chemicals	Fuel ethanol (bioethanol) carbon dioxide, glycerol, and citric acid vitamins; yeasts are also used as bioreductive catalysts in organic chemistry
Enzymes	Invertase, inulinase, pectinase, lactase, and lipase
Recombinant proteins	Hormones (e.g., insulin), viral vaccines (e.g., hepatitis B vaccine), antibodies (e.g., IgE receptor), growth factors (e.g., tumor necrosis factor), interferons (e.g., leukocyte interferon-α), blood proteins (e.g., human serum albumin), and enzymes (e.g., gastric lipase and chymosin)

microorganism known, being responsible for producing potable and industrial ethanol, which is the world's premier biotechnological commodity. However, other non-*Saccharomyces* species are increasingly being used in the production of industrial commodities (**Table 8**).

Some yeasts play detrimental roles in industry, particularly as spoilage yeasts in food and beverage production (**Table 9**). Food spoilage yeasts do not cause human infections or intoxications, but do deleteriously affect food nutritive quality and are of economic importance for food producers.

In addition to their traditional roles in food and fermentation industries, yeasts are finding increasingly important roles in the environment and in the health care sector of biotechnology. Yeasts are also invaluable as model eukaryotic cells in fundamental biological and biomedical research (**Figure 12**).

Yeasts of Environmental and Agricultural Significance

A few yeast species are known to be plant pathogens. For example, *Ophiostoma novo-ulmi* is the causative agent of Dutch Elm disease, and members of the genus *Eremothecium* cause diseases such as cotton ball in plants. On the contrary, several yeasts have been shown to be beneficial to plants in preventing fungal disease. For example, *S. cerevisiae* has potential as a phytoallexin elicitor in stimulating cereal plant defenses against fungal pathogens, and several yeasts (e.g., *Cryptococcus laurentii*, *Metschnikowia pulcherrima*, *Pichia anomala*, and *Pichia guilliermondii*) may be used in the biocontrol of fungal fruit and grain spoilage, especially in preventing postharvest

Table 8 Uses of non-*Saccharomyces* yeasts in biotechnology

Yeast	*Uses*
Candida spp.	Many uses in foods, chemicals, pharmaceuticals, and xylose fermentation (*C. shehatae*)
Kluyveromyces spp.	Lactose, inulin-fermented, rich sources of enzymes (lactase, lipase, pectinase, and recombinant chymosin)
Hansenula and *Pichia*	Cloning technology. Methylotophic yeasts (*H. polymorpha* and *P. pastoris*)
Saccharomycopsis and *Schwanniomyces*	Amylolytic yeasts (starch-degrading)
Schizosaccharomyces	Cloning technology, fuel alcohol, some beverages (rum), and biomass protein
Starmerella	Wine flavor during fermentation
Yarrowia	Protein from hydrocarbons (*Y. lipolytica*)
Zygosaccharomyces	High salt/sugar fermentations (soy sauce)

Table 9 Some yeasts important in food production and food spoilage

Yeast genus	*Importance in foods*
Candida spp.	Some species (e.g., *C. utilis*, *C. guilliermondii*) are used in the production of microbial biomass protein, vitamins, and citric acid. Some species (e.g., *C. zeylanoides*) are food spoilers in frozen poultry
Cryptococcus spp.	Some strains are used as biocontrol agents to combat fungal spoilage of postharvest fruits. *C. laurentii* is a food spoilage yeast (poultry)
Debaryomyces spp.	*D. hansenii* is a salt-tolerant food spoiler (e.g., meats and fish). Also used in biocontrol of fungal fruit diseases
Kluyveromyces spp.	Lactose-fermenting yeasts are used to produce potable alcohol from cheese whey (*K. marxianus*). Source of food enzymes (pectinase, microbial rennet, and lipase) and found in cocoa fermentations. Spoilage yeast in dairy products (fermented milks and yoghurt)
Metschnikowia spp.	*M. pulcherrimia* is used in biocontrol of fungal fruit diseases (post-harvest). Osmotolerant yeasts
Phaffia spp.	*P. rhodozyma* is a source of astaxanthin food colorant used in aquaculture (feed for salmonids)
Pichia spp.	Production of microbial biomass protein, riboflavin (*P. pastoris*). *P. membranefaciens* is an important surface film spoiler of wine and beer
Rhodotorula spp.	*R. glutinis* is used as a source of food enzymes such as lipases. Some species are food spoilers of dairy products
Saccahromyces spp.	*S. cerevisiae* is used in traditional food and beverage fermentations (baking, brewing, winemaking, etc.), source of savory food extracts, and food enzymes (e.g., invertase). Also used as fodder yeast (livestock growth factor). *S. Bayanus* is used in sparkling wine fermentations, *S. diastaticus* is a wild yeast spoiler of beer, and *S. boulardii* is used as a probiotic yeast
Schizosaccharomyces spp.	*Sch. pombe* is found in traditional African beverages (sorghum beer), rum fermentations from molasses, and may be used for wine deacidification. Regarded as an osmotolerant yeast
Schwanniomyces spp.	Starch-utilizing yeasts. *Schw. castellii* may be used for production of microbial biomass protein from starch
Yarrowia spp.	*Y. lipolytica* is used in production of microbial biomass protein, citric acid, and lipases
Zygosaccharomyces spp.	*Z. rouxii* and *Z. bailii*, being osmotolerant, are important food and beverage (e.g., wine) spoilage yeasts. *Z. rouxii* is also used in soy sauce production

fungal deterioration. Other environmental benefits of yeasts are to be found in aspects of pollution control. For example, yeasts can effectively biosorb heavy metals and detoxify chemical pollutants from industrial effluents. Some yeasts (e.g., *Candida utilis*) can effectively remove carbon and nitrogen from organic wastewater.

In agriculture, live cultures of *S. cerevisiae* have been shown to stabilize the rumen environment of ruminant animals (e.g., cattle) and improve the nutrient availability to increase animal growth or milk yields. The yeasts may be acting to scavenge oxygen and prevent oxidative stress to rumen bacteria, or they may provide malic and other dicarboxylic acids to stimulate rumen bacterial growth.

Medical Significance of Yeasts

The vast majority of yeasts are beneficial to human life. However, some yeasts are opportunistically pathogenic toward humans. Mycoses caused by *C. albicans*, collectively referred to as candidosis (candidiasis), are the most common opportunistic yeast infections. There are many predisposing factors to yeast infections, but immunocompromised individuals appear particularly susceptible to candidosis. *C. albicans* infections in AIDS patients are frequently life-threatening.

The beneficial medical aspects of yeasts are apparent in the provision of novel human therapeutic agents through yeast recombinant DNA technology (see **Table 7**). Yeasts are also extremely valuable as

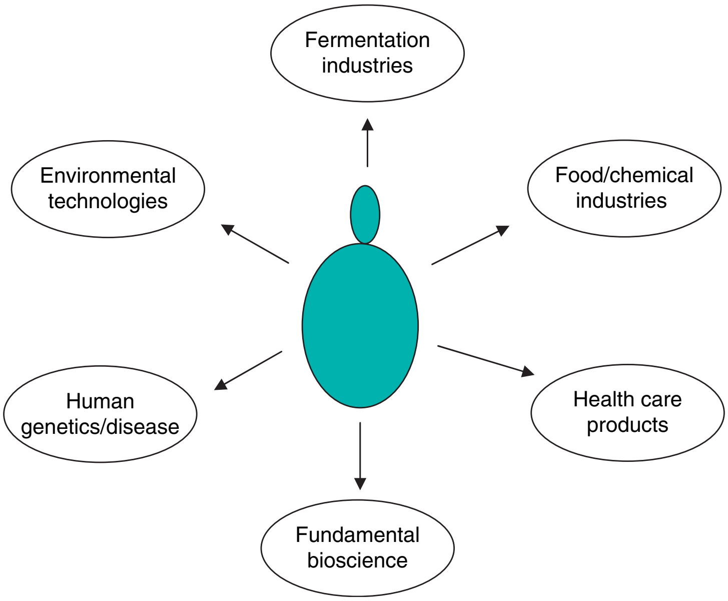

Figure 12 Uses of yeasts in biotechnology.

Table 10 Value of yeasts in biomedical research

Biomedical field	*Examples*
Oncology	Basis of cell cycle control, human oncogene(e.g., Ras) regulation; telomere function, tumor suppressor function, and design of (cyclin-dependent kinase inhibitors) CDIs/anti-cancer drugs
Aging	Mechanisms of cell aging, longevity genes, and apoptosis
Pharmacology	Multidrug resistance, drug action/metabolism, and drug screening assays
Virology	Viral gene expression, antiviral vaccines, and prion structure/function
Human genetics	Basis of human hereditary disorders and genome/proteome projects

experimental models in biomedical research, particularly in the fields of oncology, pharmacology, toxicology, virology, and human genetics (**Table 10**).

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SUBJECT INDEX

NOTES:

Cross-reference terms in italics are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space). Readers are also advised to refer to the end of each article for additional cross-references - not all of these cross-references have been included in the index cross-references.

The index is arranged in set-out style with a maximum of three levels of heading. Major discussion of a subject is indicated by bold page numbers. Page numbers suffixed by T and F refer to Tables and Figures respectively. *vs.* indicates a comparison.

This index is in letter-by-letter order, whereby hyphens and spaces within index headings are ignored in the alphabetization. For example, acid rain is alphabetized after acidosis, not after acid(s) *or* E genes are before Eggs, and not at the start of the E section. Prefixes and terms in parentheses are excluded from the initial alphabetization.

Where index subentries and sub-subentries pertaining to a subject have the same page number, they have been listed to indicate the comprehensiveness of the text.

To save space in the index the following abbreviations have been used, RSV – respiratory syncytial virus

ADCC - antibody-dependent cellular cytotoxicity
CJD - Creutzfeldt–Jakob disease
CMEIAS - Center for Microbial Image Analysis System
CMV - cytomegalovirus
EBV - Epstein–Barr virus
ED pathway - Entner-Doudoroff pathway
EMP pathway - Embden-Meyerhof-Parnas pathway
FISH - fluorescence in situ hybridization
GMOs - genetically modified organisms
HCMV - human cytomegalovirus
HHV - human herpesvirus
HPV - human papillomaviruses
HSV - herpes simplex virus
HTLV-1 - human T-cell leukemia virus 1 (human T-cell lymphotropic virus 1)
NNRTI - non-nucleoside reverse transcriptase inhibitor
NRTI - nucleoside reverse transcriptase inhibitor
ORF - open reading frame
PPK - pentose phosphoketolase
PPP - pentose phosphate
PTS - phosphotransferase system
RNP - ribonucleoprotein
SARS - severe acute respiratory syndrome
TCA - tricarboxylic acid

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